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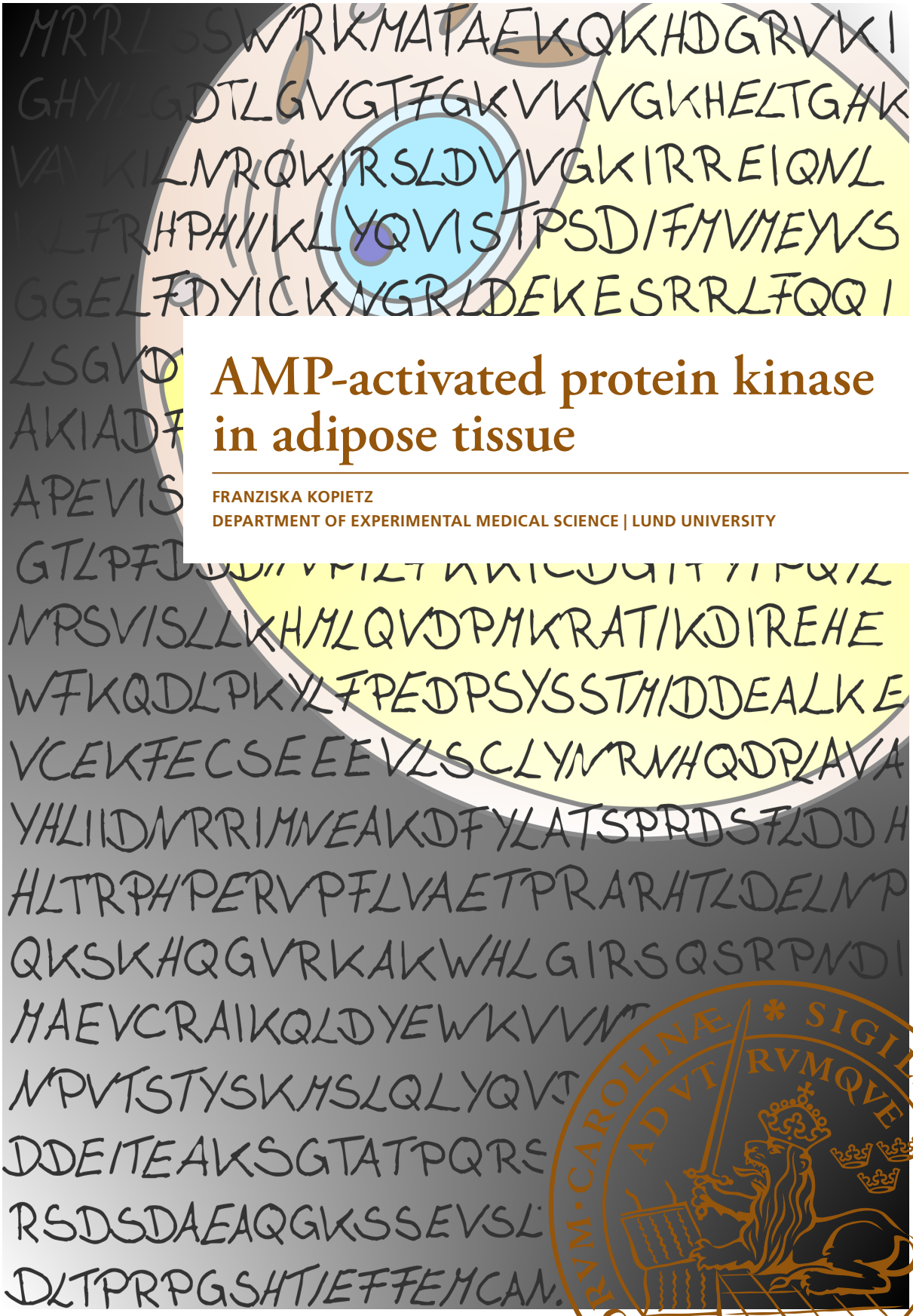

AMP-activated protein kinase in adipose tissue

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AMP-activated protein kinase in adipose tissue

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DOCTORAL DISSERTATION

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Abstract

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The first part of this work (**Paper I + II**) focuses on potential effects of AMPK activation on adipocyte metabolism. By employing AMPK activators of a new generation, A-769662 and 991, as well as a mutant mouse model, we provide evidence that AMPK activation neither affects lipolysis nor glucose uptake in human adipocytes. Thereby, our results suggest that the previously observed effects are likely to be AMPK-independent and challenge the up to now prevalent view of an anti-lipolytic and glucose uptake-inhibiting effect of AMPK activation in adipocytes.

The second part (**Paper III**) constitutes a quantitative analysis of the expression and contribution to overall AMPK kinase activity of the two regulatory AMPKβ-subunit isoforms. Moreover, we have performed a correlation analysis to investigate potential alterations in AMPK activity and expression in relation to BMI. Our results suggest that AMPKβ1 is the main isoform expressed in human adipocytes. However, it seems like AMPK activity/and expression does not correlate with human obesity/BMI.

In the last part of the work presented here (**Paper IV**), we have assessed potential mechanisms underlying the inhibitory effect of insulin on AMPK activity. While we refute the previously suggested involvement of AMPK Ser485 phosphorylation, we revealed an overall decrease in cellular energy levels in response to adipocyte insulin stimulation which might mediate the observed inhibition of AMPK activity.

Key words AMPK; adipose tissue; adipocytes; diabetes; insulin, glucose uptake; lipolysis; FA synthesis; ADaM site activators

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To my parents. *(Für meine Eltern)*

ABSTRACT

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LIST OF PAPERS

Papers included in this thesis

I. **Kopietz, F.,** Berggreen, C., Larsson, S., Sall, J., Ekelund, M., Sakamoto, K., Goransson, O. (2018). AMPK activation by A-769662 and 991 does not affect catecholamine-induced lipolysis in human adipocytes. *Am J Physiol Endocrinol Metab.* doi:10.1152/ajpendo.00110.2018

II. **Kopietz, F.,** Alshuweishi, Y., Bijland, S., Alghamdi, F., Degerman, E., Sakamoto, K., Göransson, O. (2021). A-769662 inhibits adipocyte glucose uptake in an AMPK-independent manner. *Biochem J.* doi:10.1042/BCJ20200659

III. **Kopietz, F.,** Degerman, E., Göransson, O. AMPK isoform expression patterns in adipocytes from different species and in relation to BMI. *Manuscript.*

IV. **Kopietz, F.,** Rupar, K., Berggreen, C., Sall, J., Vertommen, D., Degerman, E., Göransson, O (2020). Inhibition of AMPK activity in response to insulin in adipocytes: involvement of AMPK pS485, PDEs, and cellular energy levels. *Am J Physiol Endocrinol Metab*, 319(3), E459-E471. doi:10.1152/ajpendo.00065.2020

Additional papers by the author not included in this thesis

I. Pålbrink, A. K., **Kopietz, F.,** Morén, B., In 't Zandt, R., Kalinec, F., Stenkula, K., Degerman, E. (2020). Inner ear is a target for insulin signaling and insulin resistance: evidence from mice and auditory HEI-OC1 cells. *BMJ Open Diabetes Res Care*, 8(1). doi:10.1136/bmjdrc-2019-000820

II. Säll, J., Negoita, F., Hansson, B., **Kopietz, F.,** Linder, W., Pettersson, A. M. L., Göransson, O. (2019). Insulin induces Thr484 phosphorylation and stabilization of SIK2 in adipocytes. *Cell Signal,* 55, 73-80. doi:10.1016/j.cellsig.2018.12.011

ABBREVIATIONS

AC	adenyl cyclase
ACC	acetyl-CoA carboxylase
ACK3	acetyl-CoA carboxylase kinase-3
ACLY	ATP-citrate lyase
ADaM	allosteric drug and metabolite
AICAR	5-aminoimidazole-4-caboxamide riboside
AID	autoinhibitory domain
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ATGL	adipose triglyceride lipase
BAT	brown adipose tissue
CaMKKβ	calcium-/calmodulin-dependent kinase kinase β
cAMP	cyclic AMP
CBM	carbohydrate-binding module
ChREBP	carbohydrate response element-binding protein
CRTC2	CREB regulated transcription cofactor
CTD	C-terminal domain, C-terminal domain
DAG	diacylglyceride
FA	fatty acid
FAHFAs	esters of hydroxy fatty acids
FAS	fatty acid synthase
GAP	Rab-GTPase-activating protein
HMGCR	HMG-CoA reductase

HSL	hormone-sensitive lipase
IR	insulin receptor
IRS1	insulin receptor substrate 1/2
k/o	knockout
KD	kinase domain
LKB1	liver kinase 1 B
MAG	monoacylglyceride
MGL	monoacylglceride lipase
PDE3B	phosphodiaesterase 3B
PDE4	phosphodiesterase 4
PDK	phosphoinositide-dependent kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKA	protein kinase *A*
PKB	protein kinase B
PLIN1	perilipin 1
Rab	Ras-related in brain
scWAT	subcutaneous WAT
SVF	stromal-vascular fraction
T2D	type 2 diabetes
TAG	triacylglycerides
TCA	tricarboxylic acid
vWAT	visceral WAT
WAT	white adipose tissue
ZMP	AICAR monophosphate

BACKGROUND

Obesity and type 2 diabetes

Over the past decades, the prevalence of obesity has increased tremendously, with the number of affected people having almost tripled since 1975. According to the World Health Organization (WHO), 650 million adults and 124 million children or adolescents worldwide were obese in 2016 [1]. The main cause of obesity is an imbalance between energy intake and consumption, i.e. increased food intake in combination with decreased physical activity. Obesity is known to be a major risk factor for the development of insulin resistance which in turn is a hallmark of type 2 diabetes (T2D), a metabolic disease characterized by impaired insulin secretion as well as insulin resistance, leading to increased plasma glucose levels (hyperglycemia) [2]. Interestingly, although being a risk factor, does obesity not necessarily result in insulin resistance. Despite the fact, that most individuals with insulin resistance are overweight/obese, is only a minority of the overweight/obese individuals actually insulin resistant.[3]. A critical determinant for emerging insulin resistance during obesity is dysregulated adipose tissue, which involves inflammation as well as altered metabolism and hormone secretion of the adipocytes [4, 5].

Adipose Tissue

Mammalian adipose tissue can be divided into white adipose tissue (WAT) as well as brown (BAT)and beige adipose tissue [6]. Due to the capacity of WAT to store a large amount of energy in the form of triacylglycerides (TAG) which, if needed, can be rapidly made available again, it is a major regulator of whole-body energy homeostasis. Since the discovery of leptin [7], WAT is also regarded as an important endocrine tissue [8]. Up to now, more than 600 biochemical substances secreted by WAT have been identified and are summarized under the term adipokines [8]. Moreover, WAT serves as a protector from mechanical stress and as an insulator [6, 9]. In contrast, BAT and beige adipose tissue play a role in thermogenesis [9, 10]. However, as the work presented in this thesis is focused on WAT, the following sections are exclusively describing WAT.

WAT physiology

In humans, WAT can be divided into two specific depots: the subcutaneous adipose tissue (scWAT) and the visceral adipose tissue (vWAT), which surrounds the inner organs [11]. The majority of WAT, namely approximately 90 % of the tissue volume, is constituted of white adipocytes [9]. However, although taking up most of the WAT volume, adipocytes are greatly outnumbered by cells of the so called stromal-vascular fraction (SVF) [9, 12]. The SVF is comprised of all cells except for mature adipocytes, i.e. adipocyte precursors (preadipocytes and stem cells), fibroblasts, endothelial cells and immune cells, the latter accounting for the largest proportion [12, 13].

The white adipocytes themselves originate from fibroblast-like mesenchymal precursors, which in a complex two-step process termed adipogenesis are firstly transformed into preadipocytes, which then differentiate further into mature adipocytes [14, 15]. Mature adipocytes show a unique morphology, as 95 % of the cell is occupied by a unilocular lipid droplet [12]. In this lipid droplet, surplus energy is stored in the form TAGs. Glycerol as well as fatty acids (FAs) are synthesized from glucose taken up from the blood stream and are subsequently esterified to TAGs [12]. However, although adipocytes are capable of synthesizing FAs themselves, the bulk is directly taken up from the diet or has been synthesized from glucose in liver and transported to the adipose tissue [16, 17]. These "ready-made" FAs can then, together with glycerol, be esterified to TAGs. Upon energy demand, these TAGs can in turn be rapidly hydrolyzed, via lipolysis, to glycerol and free FAs, which are released to the circulation to provide peripheral tissues with energy [17]. This ability of adipocytes to store and release lipids upon nutritional demands highlights the importance of adipose tissue in maintaining whole body energy homeostasis.

Obesity, insulin resistance and dysregulation of WAT

Although obesity does not necessarily develop into insulin resistance, it constitutes an important risk factor [18, 19]. The exact mechanisms underlying the development of obesity-associated insulin resistance are not understood but adipose tissue dysregulation has been identified as an important factor.

It was shown that during obesity, the main metabolic pathways of adipocytes i.e. FA synthesis and lipolysis, but also glucose uptake, are altered. Although the amount of glucose taken up by adipocytes is only minor compared to cells of other tissues, there is evidence for adipocyte glucose uptake playing an important role in the regulation of whole-body insulin resistance. As summarized by Sherpard & Kahn in 1999, it was by then already known that the expression of the glucose transporter GLUT4 in adipocytes is impaired in human obesity as well as T2D [20]. Moreover, by employing adipose tissue GLUT4 deficient mice, it was proposed that downregulation of GLUT4 and

with that glucose transport into adipocytes is indeed associated with impaired insulin sensitivity in liver and muscle [21]. Conversely, adipose specific overexpression of GLUT4 was shown to lead to reduced fasting glycemia and enhanced glucose tolerance [22]. This positive effect of increased glucose uptake is proposed to be due to induced expression of lipogenic genes in response to glucose [23]. In general, increased release of FAs from adipose tissue through lipolysis is associated with poor metabolic health and increasing insulin resistance due to fat accumulation in ectopic, insulin-responsive tissue [5, 24, 25]. However, a study in high fat diet fed mice suggests that inhibition of lipolysis causes a shift in the lipid metabolism towards increased FA synthesis in adipocytes, inducing improved whole-body glucose metabolism and insulin sensitivity [25]. Adipocytes are capable of synthesizing a specific type of FAs termed fatty acid esters of hydroxy fatty acids (FAHFAs) with favorable metabolic effects like enhanced insulin sensitivity in adipocytes and anti-inflammatory effects [26, 27]. Taken together, this also highlights the importance of glucose uptake, FA synthesis and lipolysis in maintaining whole-body insulin sensitivity.

Apart from the already mentioned FAHFAs, the secretion of other adipokines including adiponectin, has also been shown to be associated with increased insulin sensitivity and to be altered in obesity [26, 28, 29]. Moreover, obesity induces the secretion of pro-inflammatory cytokines and chemokines, causing the infiltration of adipose tissue with macrophages, which further drive the creation of an inflammatory milieu [2, 30-32]. The attracted cytokines are suspected to directly cause adipose tissue dysfunction by impairing insulin signaling, glucose uptake and lipogenesis and enhancing lipolysis [33].

Insulin-regulated adipocyte metabolism

Insulin is one of the major regulators of adipocyte metabolism. As shown in Figure 1, binding of insulin to the insulin receptor (IR) on the cell surface induces IR autophosphorylation and subsequent phosphorylation of insulin receptor substrate 1 (IRS1). Activation of IRS1 is followed by activation of phosphatidylinositol-3-kinase (PI3K) which catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), inducing the activation of phosphoinositide-dependent kinase 1 (PDK) as well as the translocation of Akt, also known as protein kinase B (PKB), to the plasma membrane. Together, this leads to the activation of Akt via phosphorylation at Thr308 by PDK1 and Ser473 by the mammalian target of rapamycin complex 2 (mTORC2) [34, 35]. Once active, Akt phosphorylates several downstream proteins, thereby regulating carbohydrate and lipid metabolism [36].

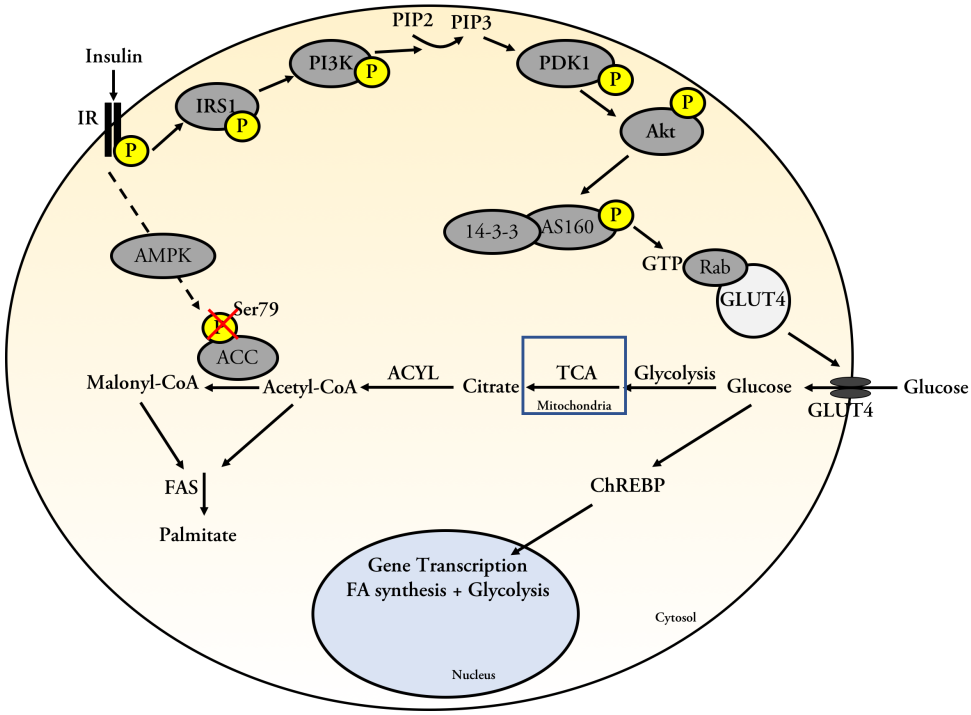

Figure 1. Stimulation of FA synthesis by insulin – overview.

Binding of insulin to the insulin recptor (IR) leads to autophosphorylation and activation of the recptor resulting in phosphorylation of IRS1 and its recruitment to the plasma membrane. Subsequently, IRS1 phosphorylated and activates PI3K which then catalyzed the converstion of PIP_2 to PIP_3 leading to phosphorylation and activation of PDK1 resulting in phosphorylation and translocation of Akt to the plasma membrane. Activated Akt then phosphorylates the Rab-GAP domain containing protein AS160 leading to the inhibition of AS160 GAP activity allowing the loading of Rab, loacted in the GLUT4 vesicle membrane, with GTP which induces the translocation of GLUT4 to membrane. It is not entirly understood yet how AS160 induces the translocation of the GLUT4 vesicles to the plasma membrane but it is suggested to involve the binding of the scaffold protein 14-3-3 to AS160. Glucose entering the adipocyte via the GLUT4 transporter is converted to citrate via glycolysis and the TCA cycle. Upon entry into the cytoplasma from the mitochondria, ACLY convertes citrate to acetyl-CoA which is then converted to malonyl-CoA by ACC. Subsequently, malonyl CoA and acetyl CoA cen be utilized for the synthesis of palmitate by FAS. Additionally, FA synthesis is further promoted via the activation of ChREBP by glucose inducing the transcription of genese required fo glycolysis and FA synthesis. Moreover, ACC can be allosertically inhibited by citrate as well as insulin-induced reduction of the inhibitory phosphorylation of ACC at Ser79, which is suggested to be AMPK-dependent.

Glucose uptake in adipocytes

The key step for insulin-stimulated glucose uptake in adipocytes (Figure 1) is the translocation of the glucose transporter GLUT4 from its intracellular vesicles to the plasma membrane [37]. Trafficking of these vesicles depends on the GTPase Ras-related in brain (Rab) protein, located in the vesicle membrane, which needs to be converted from its inactive (GDP-bound) state to its active (GTP-bound) state to allow translocation of the GLUT4-containing vesicles [38]. This activation of Rab has been shown to involve the Rab-GTPase-activating protein (GAP) domain containing protein Akt substrate of 160 kDa (AS160) also known as TBC1D4 [39, 40]. In

response to insulin-induced Akt activation, AS160 becomes phosphorylated at multiple residues, which is accompanied by inactivation of its GAP activity which in turn allows GTP-loading and activation of Rab [41]. The exact mechanism by which AS160 induces translocation of the GLUT4 vesicles is not understood. However, there is evidence for the association of AS160 with the scaffold protein 14-3-3, proposed to be mediated by insulin-induced AS160 Ser341 and Thr642 phosphorylation, to be a requirement [42, 43].

FA synthesis

FA synthesis requires the coordinated activity of several enzymes, summarized in Figure 1 [44, 45]. Firstly, the production of citrate from glucose, via glycolysis and the tricarboxylic acid (TCA) cycle, is required. Upon entry into the cytosol, ATP-citrate lyase (ACLY) converts citrate to acetyl-CoA. To initiate the synthesis of FAs, acetyl-CoA needs to be converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). The final enzyme required is the fatty acid synthase (FAS), which catalyzes the synthesis of palmitate in a multi-step process starting from acetyl-CoA, which is then step by step elongated via the addition of malonyl-CoA. Therefore, malonyl-CoA constitutes an ultimate requirement for FA synthesis, making the synthesis of malonyl-CoA by ACC the rate limiting step in this process. The activity of ACC is on the one hand allosterically regulated by citrate, shown to induce activation, and possibly also acyl-CoA esters, suggested to cause an inhibition [46]. On the other hand, ACC is regulated via phosphorylation as described in more detail below.

Insulin stimulates FA synthesis in different ways (Figure 1) [44, 45]. Firstly, by promoting glucose uptake, insulin provides the necessary substrate. Moreover, intermediates of glucose metabolism have been shown to induce the carbohydrate response element-binding protein (ChREBP), a transcription factor inducing the expression of genes required for glycolysis and FA synthesis [47]. Furthermore, studies by us and others have shown that insulin induces ACC activity by reducing the inhibitory ACC Ser79 phosphorylation, an effect shown to be Akt dependent [48-50]. The mechanism by which insulin reduces ACC Ser79 phosphorylation is unknown but is suspected to involve the AMP-activated protein kinase (AMPK), which so far is the only kinase shown to phosphorylate ACC at Ser79 [51-54]. However, if this insulin-induced inhibition of ACC Ser79 is a requirement for the induction of FA synthesis by insulin at all, is also not known.

Lipolysis

Lipolysis describes the sequential hydrolysis of TAGs stored in the adipocyte lipid droplet to free FAs and glycerol (Figure 2). Major activators of this process are catecholamines, which through binding to the β-adrenergic cell surface receptor induce the activation of adenyl cyclase (AC), which converts ATP to cyclic AMP (cAMP) [55,

56]. The increasing levels of cAMP activate protein kinase A (PKA), initiating the phosphorylation of the lipid-droplet-associated protein perilipin 1 (PLIN1) and the cytoplasmic hormone-sensitive lipase (HSL). Following PKA activation, HSL as well as the adipose triglyceride lipase (ATGL) are translocated from the cytoplasm to the lipid droplet surface [57]. In general, the lipolytic cascade proceeds as follows: firstly, ATGL hydrolyses TAG to diacylglycerides (DAGs), which are then further hydrolyzed by HSL to monoacylglycerides (MAGs) before the final hydrolyzation by monoacylglyceride lipase (MGL), resulting in free FAs and glycerol which are released to the circulation [55]. However, there is evidence that ATGL is neglectable for stimulated lipolysis in human adipocytes and that HSL also catalyzes the rate-limiting step of TAG to DAG hydrolysis in these cells [58, 59].

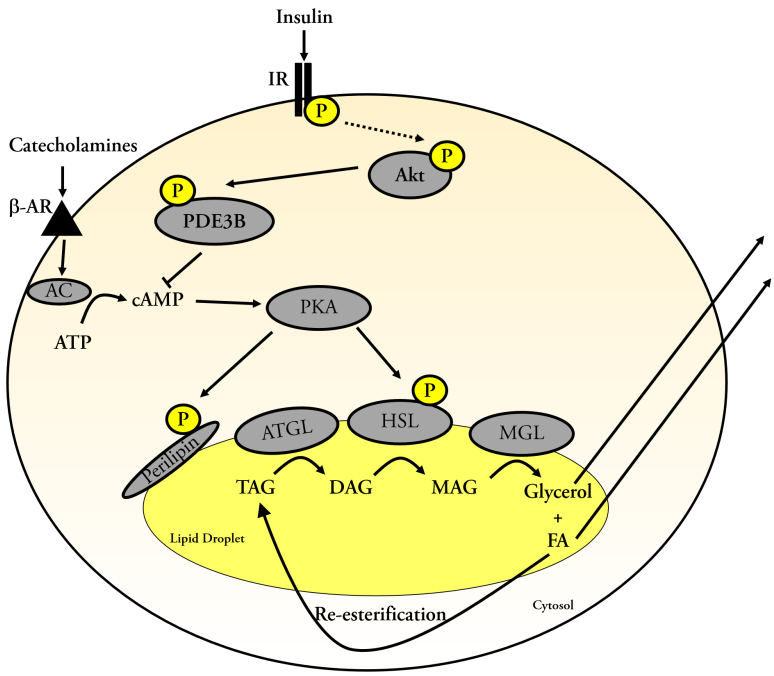

Figure 2. Regulation of Lipolysis by catecholamines and insulin.

Binding of catecholamines to the β-adrenergic receptor (β-AR) leads to the activation of AC, which catalyzes the conversion of ATP to cAMP. An increase in cellular cAMP levels causes activation of PKA which phosphorylates PLIN1 as well as HSL leading to the translocation of ATGL and HSL to the surface of the lipid droplet, inducing the lipolytic cascade. ATGL hydrolyzed the TAG stored in the lipid droplet to DAG which is then hydrolyzed to MAG by HSL followed by the hydrolysis of MAG to glycerol and three FAs. Glycerol and the FAs are then exported and released into the circulation. Alternatively, under energy consumption the FAs can be re-esterified to TAG. Insulin induces the phosphorylation of PDE3B in an Akt-dependent manner leading to the breakdown of cAMP causing an inhibition PKA activity thereby reducing lipolysis.

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Activity of HSL itself is regulated via phosphorylation at different sites. Upon catecholamine stimulation, HSL becomes phosphorylated by PKA at three different sites: Ser563, Ser659 and Ser660 [60-62]. While initially, Ser563 was thought to be the main activity regulating site, it was later shown that phosphorylation of this site is no ultimate requirement for activation and that Ser659 and Ser660 are the main sites regulating HSL activity, at least *in vitro* [60]. In addition to these PKA sites, HSL was also shown to be phosphorylated at Ser565 by AMPK [63]. Phosphorylation at Ser565 is proposed to prevent PKA-mediated Ser563 phosphorylation due to steric hindrance, thereby exerting an anti-lipolytic effect [63-65].

The main negative regulator of lipolysis is insulin. By activating Akt, insulin induces an Akt-dependent phosphorylation of phosphodiesterase 3B (PDE3B), which catalyzes the hydrolysis of cAMP to AMP, thereby leading to an inactivation of PKA (Figure 2) [56, 66]. However, the mechanism by which insulin inhibits lipolysis is not fully understood and still debated [56].

AMP-activated protein kinase

In the 1980s, a kinase inhibiting ACC activity through phosphorylation was discovered [67]. At that time, this kinase was named acetyl-CoA carboxylase kinase-3 (ACK3). Later on, it was discovered that ACK3 activity could be stimulated by AMP and that it not only phosphorylates ACC but is also responsible for the phosphorylation of HMG-CoA reductase (HMGCR) [68]. Altogether, this led to the renaming of ACK3 to AMPK by Munday et al. in 1988 [69]. Since then, AMPK has attracted increasing attention as a drug target, as it was found to be activated in response to many cues associated with improved health like caloric restriction and exercise [70, 71]. Moreover, AMPK was suggested to be involved in the positive effects of many natural products like berberine and resveratrol as well as existing drugs like salicylate and metformin [72-75].

Structure of AMPK

AMPK is a heterotrimeric protein composed of a catalytic α-subunit and the two regulatory subunits β and γ [76]. In vertebrates, each subunit exists in different isoforms, with two each for the α- and β-subunit and three for the γ-subunit. In theory, this allows for the composition of 12 different AMPK complexes, but it seems like certain isoform combinations are favored dependent on the tissue and species.

Starting from the N-terminus, the α-subunit consists of a Ser/Thr kinase domain (KD) containing the activation loop/Thr-loop, an autoinhibitory domain (AID), the α-linker segment and a globular C-terminal domain (α-CTD), which contains a Ser/Thr-loop (Figure 3A) [77, 78]. All of the parts included in the catalytic α-subunit are in one way or the other involved in regulating AMPK activity. Included in the activation-loop of the KD is the phosphorylation site Thr172, which plays a critical role in the regulation/activation by upstream kinases (described in more detail further down) [79]. Further phosphorylation sites with a possible role in the regulation of AMPK activity are found in the Ser/Thr loop of the α-CTD, however these sites are much less studied than Thr172 [80-82]. The AID domain possesses its name due to the discovery that its binding to the isolated KD domain reduces the kinase activity about 10-fold [83, 84]. Finally, the α-linker is important for the regulation of activity by nucleotides, which however bind to the γ-subunit [85].

The N-terminus of the AMPK β-subunit undergoes posttranslational myristoylation (Figure 3A), a modification proposed to be involved in the phosphorylation of AMPKα Thr172 [86]. Additionally, the β-subunit possesses a carbohydrate-binding module (β-CBM,; Figure 3A). The exact function of the β-CBM is not understood, but it was shown that a part of it is involved in the formation of a binding pocket for small ligands

together with the α-KD [87]. This binding pocket is also referred to as the allosteric drug and metabolite (ADaM) binding site and comprises a AMPK β-CBM phosphorylation site at Ser108, at least in β1-containing complexes. C-terminally of the β-CBM lies the C-terminal domain (CTD), acting as a scaffold for the formation of the heterotrimeric complex, as it interacts with the α- as well as the γ-subunit [88, 89].

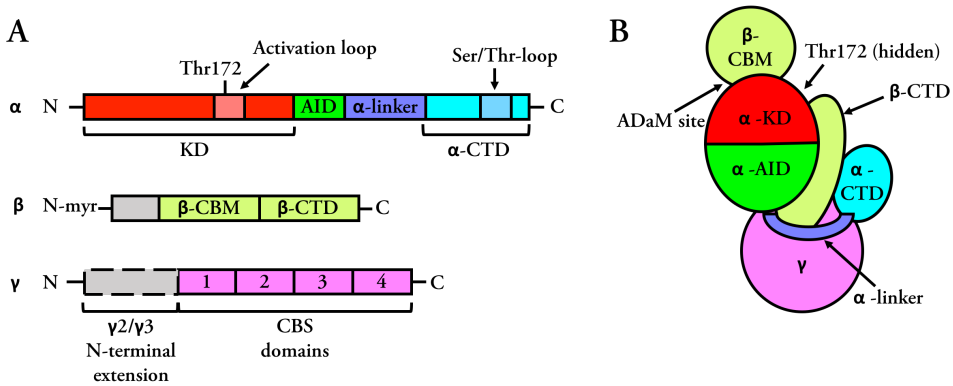

Figure 3. AMPK Structure
(A) Domain structure of the three AMPK subunits α, β and γ. (B) Arrangement of the AMPK heterotrimeric complex.

The γ-subunit contains four tandem repeats of a cystathionine β-synthase (CBS) domain at the C-terminus (Figure 3A), which shape four binding sites for the regulatory nucleotides AMP, ADP and ATP. However, it appears that only three of these sites actually bind nucleotides [89]. The N-terminus of the γ-subunit varies greatly among the three isoforms as the γ2- and γ3-subunit contain a N-terminal extension which is not present in the γ1-subunit (Figure 3A) [90].

The arrangement of all three subunits in the heterotrimeric AMPK complex is depicted in Figure 3B.

Regulation of AMPK

The early finding of AMPK activity being regulated by cellular energy levels was later complemented with the discovery that AMPKα Thr172 phosphorylation in the T-loop of the α-KD plays a pivotal role in the AMP-induced AMPK activation [68, 79, 91]. The main upstream kinases involved in the phosphorylation of AMPKα Thr172 are the calcium-/calmodulin-dependent kinase kinase β (CaMKKβ) and liver kinase 1 B (LKB1) [92-95]. The phosphorylation status of AMPKα Thr172 is dependent upon the identity of the nucleotide (AMP, ADP or ATP) bound to the γ-subunit. In the

event of low cellular energy levels, binding of AMP or ADP to the CBS3 induces phosphorylation of AMPKα Thr172 by LKB1, but not CaMKKβ, and also prevents dephosphorylation by hindering phosphatases from accessing Thr172 [96-98]. A further mechanism by which AMP, not ADP though, can activate AMPK is by binding to CBS1 and thereby inducing allosteric activation [98, 99]. It seems like, out of the four CBS domains available at the γ-subunit, only CBS1+3 have a regulatory function, while CBS2 appears to always be empty and CBS4 harbors a tightly bound AMP molecule [89, 98]. However, it is also important to mention here that there is evidence that AMPK complexes containing different γ-isoforms possess different physiological functions and/or cellular localization and that their regulation by the nucleotides might also vary accordingly [100].

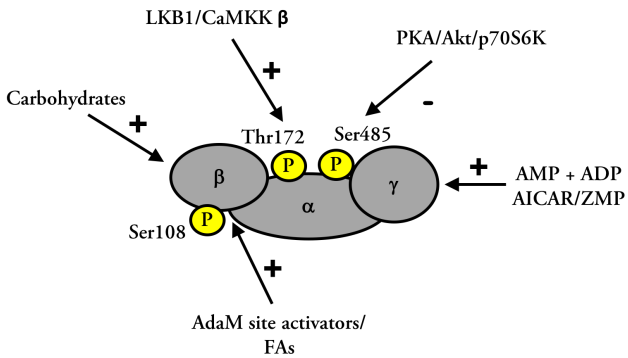

Figure 4. Regulators of AMPK activity.
AMPK activity has been shown to be regulated in different ways. Reduced cellular energy levels lead to the binding of AMP and/or ADP to the γ-subunit leading to alloseric activation as well as increased phosphorylation of AMPKα at Thr172 by the upstream kinase LKB1. Phosphorylation at the activity-regulating site Thr172 is also induced by CaMKKβ in response to Ca^{2+}. Moreover, it has been shown that AMPK can be activated by different pharmacological activators like AICAR, which is converted to ZMP which acts as an AMP-mimetic and binds to the γ-subunit and more specific activators like A-769662 and 991 which bind to the ADaM site. Activation of AMPK by binding of ligands to the ADaM site requires the phosphorylation of AMPKβ at Ser108, an autophosphorylation site. Additionally, it has been suggested that AMPK can be activated by energy substrates like carbohydartes, binding to the β-CBM, and FAs, binding to the ADaM site also requiring Ser108 phosphorylation. Phosphorylation of AMPK at Ser485 by PKA, Akt as well as p70S6K as been suggested to cause an inhibition of AMPK activity.

Although regulation of AMPK activity by cellular energy levels via binding of nucleotides to the γ-subunit and AMPKα Thr172 phosphorylation constitutes the best-defined mechanism, additional mechanisms are emerging (summarized in Figure 4). For example, it has been shown that AMPKα phosphorylation at the Ser/Thr loop of the α-CTD by upstream kinases potentially plays a role in the regulation of AMPK activity [48, 80-82, 101-103]. One of the suggested phosphorylation sites is Ser485, a site shown to be phosphorylated by PKA, p70S6K, as well as Akt, the latter being topic of **Paper IV** presented in this thesis [48, 80-82, 101-103]. Furthermore, there is evidence for AMPK being directly regulated by the availability of energy substrates e.g.

via the binding of carbohydrates to the β-CBM or binding of lipids to the ADaM site [104, 105]. Describing all the suggested regulatory mechanisms suggested to be involved in the regulation of AMPK activity would exceed the scope of this thesis, but further mechanisms described involve ubiquitination of AMPK, as well as changes in protein-protein interactions, subcellular localization and modification of upstream kinases [106].

Pharmacological activators

As mentioned previously, AMPK activation is associated with many beneficial health effects, for example in relation to insulin resistance and T2D. In fact, metformin, the first line drug for the treatment of T2D, is suggested to mediate its positive effects, i.e. reduced gluconeogenesis, via activation of AMPK as a result of changes in cellular energy levels [75, 107-109]. Therefore, much focus was put on the development of AMPK activators in recent years. In theory, AMPK can be activated by any agent inducing an increase in the cellular AMP/ADP:ATP ratio as observed for many natural compounds as well as metformin. However, the focus of this section will be on activators, which alter the kinase activity by directly binding to AMPK. Historically, the most commonly used such activator is 5-aminoimidazole-4-caboxamide riboside (AICAR), which is converted to the AMP-mimetic AICAR monophosphate (ZMP) upon entry into the cell and binds to the γ-CBS3 (Figure 4) [110, 111]. However, AMPK is not the only enzyme regulated via AMP [112, 113]. Therefore, AICAR, as well as other activators altering cellular energy levels, are rather unspecific. In the case of AICAR, many observed effects have already been shown to be AMPK-independent with the help of animal models and more specific activators as also demonstrated in **Paper I + II** [96, 114-117]. A newer generation of more specific AMPK activators are the ADaM site activators, which interact with the pocket formed by the α-KD and β-CBM. With A-769662, identified in 2006, being the first activator of this kind, many more followed, including 991, MK-8722, PF-06409577 and SC4, just to name a few [87, 118-121]. A common feature of these ADaM site activators is the requirement of AMPKβ1 Ser108 phosphorylation (Figure 4), located within the ADaM site, for the compounds to bind [87, 122]. This requirement of AMPKβ1 Ser108 phosphorylation was also exploited in **Paper II** to investigate AMPK-dependency of effects observed with A-769662. All ADaM site activators identified to date were proven to have a preference for β1-containing complexes, although the magnitude of this preference varies largely among the activators [123]. The furthest these described AMPK activators have come towards clinical studies is the use in preclinical non-human primate models (PF-06409577 and MK-8722). Concerning the development of AMPK activators as drugs, so far none of the listed activators has made it to clinical trials. There is one AMPK activator, O304, which has been used in Phase IIa clinical trials showing promising results with regard to the treatment of diabetes [124]. However, the exact mechanisms by which this compound activates AMPK are not understood.

AMPK in muscle and liver – biological role and therapeutic target for T2D

Due to its ability to sense cellular energy levels, AMPK is often referred to as a cellular "fuel gauge" [125]. However, AMPK cannot only sense the energy levels but also alters metabolism in a way that cellular energy homeostasis is maintained. This means, in the event of low energy levels when AMPK is active, it functions to shut down energy-consuming, anabolic pathways, while energy-generating, catabolic pathways are promoted. Since its discovery, AMPK has been shown to be involved in the regulation of diverse metabolic pathways which also led to the revelation of its potential for the treatment of a variety of diseases. Due to their importance in regulating whole glucose homeostasis, AMPK activation has been extensively studied in muscle and liver tissue and obtained results propose promising effects with potential use for the treatment of T2D. These promising effects (described in more detail in the upcoming sections) include improved glucose uptake in muscle as well as decreased lipid accumulation and glucose output (gluconeogenesis) in the liver. Moreover, AMPK was found to be activated in response to several factors associated with an improved health status and insulin sensitivity, like exercise and caloric restriction [106]. Taken together, these findings propose AMPK activation as a valuable therapeutical strategy for the treatment of T2D.

AMPK in liver

The first function of AMPK discovered was the inhibition of lipid storage via suppression of FA as well as cholesterol synthesis in hepatocytes [68]. The importance of AMPK in regulating liver FA metabolism is well established today and has been confirmed with the help of AMPK knockout (k/o) animals [126]. AMPK removal form hepatocytes was shown reduce FA oxidation and increase FA synthesis, while increased expression of AMPK was shown to reduce the expression of lipogenic genes leading to reduced lipid accumulation [127-129]. The effects of AMPK on liver lipid metabolism are suggested to be due to subsequent changes in ACC phosphorylation at the AMPK site Ser79 (ACC1) and Ser212 (ACC2). This involvement of ACC was confirmed with the help of ACC phosphorylation mutant as well as the employment of an ACC inhibitor [130, 131]. Although still debated, AMPK was also thought to play a role in the regulation of hepatic glucose production. These initial findings proposing a role of AMPK were based on the use of AICAR and were later on refuted with the help of liver specific genetic mouse models and the more specific activator A-769662 [126]. However, there is evidence that AMPK might be able to regulate gluconeogenesis by causing transcriptional alterations via CREB regulated transcription cofactor (CRTC2) and class IIA histone deacetylases (HDACs) [114, 123, 132-134]. Moreover, AMPK was found to counteract the stimulatory effect of glucagon on gluconeogenesis which is proposed to be due to phosphorylation of phosphodiesterase 4 (PDE4) by AMPK, antagonizing the accumulation of cAMP [135]. AMPK-induced alterations in liver

gluconeogenesis are also believed to be the basis for the beneficial effects of metformin in the treatment of T2D [75, 126]. Although the involvement of AMPK in mediating metformin effects is still strongly debated, it seems like there is evidence for AMPK involvement under the condition that insulin resistance is already established and that physiological metformin concentrations are used in the studies [108, 126, 136].

AMPK in muscle

In muscle, early studies proposed an induction of glucose uptake as well as FA oxidation in response to AMPK activation [137]. However, this conclusion was made based on observations with AICAR. Studies with contraction-induced AMPK activation have remained inconclusive so far, suggesting, if anything, only a partial requirement of AMPK in contraction-induced increase of muscle glucose uptake and FA oxidation [138]. Investigations for mechanisms involved in the potential regulation of these pathways by AMPK propose an induction of GLUT4 translocation to the plasma membrane due to AMPK-induced phosphorylation of TBC1D1, Rab-GAP expressed in muscle, at several sites [138]. Furthermore, it was shown that muscle insulin sensitivity, measured as increased GLUT4 translocation, is increased post-exercise and that this occurs independently of the proximal insulin signal cascade [138, 139]. There is evidence that AMPK plays an important role in inducing this increased insulin sensitivity by direct phosphorylation of AS160 at Thr642 and Ser704 [140-142].

AMPK in WAT

In contrast to liver and muscle, AMPK in adipose tissue is only studied to a limited extent. However, given the central role of adipose tissue dysfunction in the development of insulin resistance together with the role of AMPK as a master regulator of cell metabolism the investigation of AMPK also in adipose tissue is clearly warranted [21, 26]. In fact, it was discovered that deletion of AMPK in adipose tissue induces lipid accumulation and insulin resistance in liver, highlighting the importance of adipocyte AMPK in maintaining insulin sensitivity [116]. However, to date the physiological role of AMPK in adipose tissue is not completely understood. Additionally, in view of AMPK activators being used as potential drugs for the treatment of metabolic diseases like T2D, potential effects need to be carefully evaluated, especially in metabolically and hormonally active tissues like the adipose tissue.

AMPK expression and activity

Up until now, the exact composition of the AMPK complex(es) in adipose tissue remains elusive. While scientists agree upon AMPKα1 being the main catalytic subunit, the expression pattern of the two regulatory subunits is only poorly characterized [64, 143]. Regarding the β-subunit expression, it is proposed that β2 is the predominant isoform in all of these adipocyte models [144] and has also been investigated in **Paper III**. Determination of the γ-isoforms expressed in adipose tissue, and in general, has proven to be challenging so far, due to the existence of several different splice variants and the lack of specific antibodies. However, mRNA analyses point towards γ1 and γ2 being present in adipocytes ([145] and personal communication David Carling). The expression of γ3 appears to be unique for muscle tissue [90, 145].

Studies from morbidly obese human subjects showed decreased AMPK activity, measured as decreased AMPK Thr172 phosphorylation, in adipose tissue of insulin resistant subjects compared to BMI-matched insulin sensitive subjects [146]. Moreover, it was shown that AMPK activity in scWAT is significantly increased post-bariatric surgery [147, 148]. Importantly, these studies were performed on adipose tissue rather than on isolated adipocytes. Therefore, it remains unclear if the observed changes are due to alterations in the adipocytes themselves or if they are caused by cells other than adipocytes present in WAT, an issue which was also addressed in **Paper III**.

Regulation of AMPK in adipocytes

Apart from the knowledge that AMPK activity is regulated in response to cellular energy levels, several factors triggering AMPK activation in adipose tissue have been identified. For example, it was shown that WAT AMPK is activated in response to stresses like exercise and fasting, which at the same time suggests an involvement of increasing cAMP levels [64, 106, 149, 150]. Indeed, AMPK activity has been shown to be stimulated in white adipocytes in response to cAMP-elevating agents, like isoproterenol [64, 65, 151-153]. The exact mechanism underlying this activating effect is unclear but it has been suggested to involve PDE4, Epac, PKA as well as lipolysis [151, 152]. The activating effect of lipolysis on AMPK activity is proposed to be caused by increased AMP:ATP due to increased, energy-consuming, re-esterification of lipids [151]. Although many studies point towards PKA being involved in the activation of AMPK there is one study proposing inhibition of AMPK by PKA via phosphorylation at AMPK Ser173 [154]. In addition, the induction of AMPK activity by catecholamines was shown to be reduced in response to insulin stimulation, proposed to be mediated via a PDE3B/Akt dependent mechanism [152]. Moreover, insulin has not only been shown to inhibit catecholamine-induced AMPK activation but also basal AMPK activity. An earlier study from our lab showed that this inhibition of basal activity occurs in an Akt-dependent manner, which at the same time was associated with increased AMPK Ser485 phosphorylation (Figure 5A) [48]. If the

phosphorylation of AMPK Ser485 is required for the insulin-induced inhibition of AMPK activity remained unknown so far, a question we addressed in **Paper IV.**

Regulation of glucose uptake in adipocytes

Up until the publication of **Paper II** of this thesis, the role of AMPK in regulation of adipocyte glucose uptake was mainly studied with AICAR, leading to varying results. In 3T3-L1 adipocytes, AMPK activation by AICAR was reported to increase basal and decrease insulin-stimulated glucose uptake without any changes in early insulin signaling [155]. Instead, the decrease in insulin-stimulated glucose uptake was proposed to be due to reduced translocation of the glucose transporter GLUT4 to the plasma membrane [155]. One year later however, the stimulating effect of AMPK activation on basal glucose uptake was questioned by another study employing AICAR in the same cell model, as the effect of AICAR on glucose uptake was maintained in cells expressing a kinase inactive Thr172Ala AMPK mutant [156]. This result suggested that the effect on basal glucose uptake occurred independently of AMPK [156]. Later on, investigations in primary rat adipocytes with AICAR proposed an inhibitory effect of AMPK activation on basal as well as insulin-stimulated glucose uptake in these cells [52].

The exact mechanism by which AICAR-induced AMPK activation might reduce insulin-stimulated glucose uptake are unclear to date. In rat adipocytes, AICAR stimulation was shown to be accompanied by reduced AS160 Thr642 phosphorylation and with that, impaired translocation of GLUT4 to the plasma membrane [157]. This effect of AICAR was abolished by the expression of a kinase dead AMPKα1 mutant. Although AS160 is phosphorylated at multiple sites, GLUT4 translocation has been proposed to be mainly dependent upon Ser341 and Thr642 phosphorylation [39, 42]. Both of these sites seem to be poor substrates for AMPK, which has only been shown to phosphorylate AS160 at Ser588 [42]. However, the role of this phosphorylation site remains elusive so far and so does the mechanism by which AMPK activation could prevent AS160 Thr642 phosphorylation.

Interestingly, a study by Choi et al. employing adipose tissue specific AMPKα1/α2 k/o mice suggests that AMPK plays only a minor role in the regulation of whole-body glucose metabolism [158]. This conclusion was made based on the observation that the k/o mice did not display any alterations in fasting blood glucose or glucose- and insulin tolerance. However, as this study was performed with mice on chow diet, no conclusion can be drawn regarding a potentially positive role of adipocyte AMPKα1/α2 deficiency in the prevention of metabolic effects induced by caloric overload. This is also a difference to the previously mentioned study in which AMPK deletion led to increased lipid accumulation in liver as that observation by Mottilo et al. was made in mice on high-fat diet [116]. Furthermore, in the study by Choi et al. adipocyte glucose uptake

was not assessed and it remains unknown whether adipocyte AMPKα1/α2 deficiency directly affects adipocyte glucose uptake.

Regulation of FA synthesis and lipolysis

The earliest studies on AMPK revealed that AMPK activation suppresses FA synthesis in liver via increased phosphorylation of ACC at Ser79, leading to decreased ACC activity and malonyl CoA synthesis. Subsequent studies propose a role of AMPK also in the regulation of adipocyte FA synthesis by showing decreased ACC activity in response to AICAR as well as exercise or via the expression of constitutively active AMPK [52, 64, 111, 159]. Furthermore, prolonged stimulation of adipocytes with AICAR or leptin, also shown to increase AMPK activity, are suggested to decrease lipid storage via altered gene expression [160, 161]. The role of AMPK in the regulation of FA synthesis is also further highlighted by the fact that ACC Ser79 is the main site required for the regulation of ACC activity and that AMPK is the only kinase shown so far to be required for ACC Ser79 phosphorylation [51, 69]. Additionally, a study from our laboratory showed that insulin stimulation of adipocytes leads to a Akt-dependent decreased AMPK activity causing a reduction in ACC Ser79 phosphorylation (Figure 5A), which is further confirmed by the data presented in **Paper IV** [48]. However, to date, the relation between AMPK and adipocyte FA synthesis is only poorly studied and especially not with the help of more specific activators than AICAR or animal models. Therefore, it remains unclear whether this decrease in ACC Ser79 phosphorylation is a requirement for the insulin-induced stimulation of adipocyte FA synthesis. However, a recent study employing ACC1/2 Ser79Ala/Ser212Ala knockin mice suggests that Akt plays a key role in the insulin-stimulated FA synthesis by reducing ACC Ser79 phosphorylation [162].

The other main function of adipocytes, along with lipid storage, is the release of FAs through lipolysis. AMPK activation is commonly viewed as having an anti-lipolytic effect in adipocytes (Figure 5B), a view which is questioned by **Paper I** of this work. This view is based on findings with AICAR, which propose an inhibitory effect of AMPK activation on basal as well as stimulated lipolysis in primary rat adipocytes [64, 111, 163]. Furthermore, as mentioned previously, AICAR also reduces HSL activation, which is suggested to be due to AMPK-induced HSL Ser565 phosphorylation, hindering PKA from phosphorylating HSL Ser563 and Ser660 (Figure 5B) [63-65]. Interestingly however, in a study performed in 3T3-L1 adipocytes, it was shown that if PKA stimulation, and with that HSL Ser563 and Ser660 phosphorylation, precedes AICAR stimulation and HSL Ser565 phosphorylation, HSL phosphorylation at Ser660 could be maintained, in contrast to Ser563 [150]. This observation indicates an important role of HSL Ser660 phosphorylation in the regulation of HSL activity, which could prevent AMPK-induced HSL inhibition. Moreover, decreased basal as well as stimulated lipolysis in response to AICAR as well as adiponectin, is also suggested to occur in human adipocytes [164]. In contrast to the predominantly anti-lipolytic effect

reported, an early study in 3T3-L1 adipocytes claims that AMPK is required for maximal lipolysis [153]. Furthermore, chronic AICAR treatment is proposed to change adipocyte lipid metabolism from lipid storage towards energy dissipation through induced FA oxidation [160, 165]. This alteration is thought to be due to increased expression of ATGL and PLIN1.

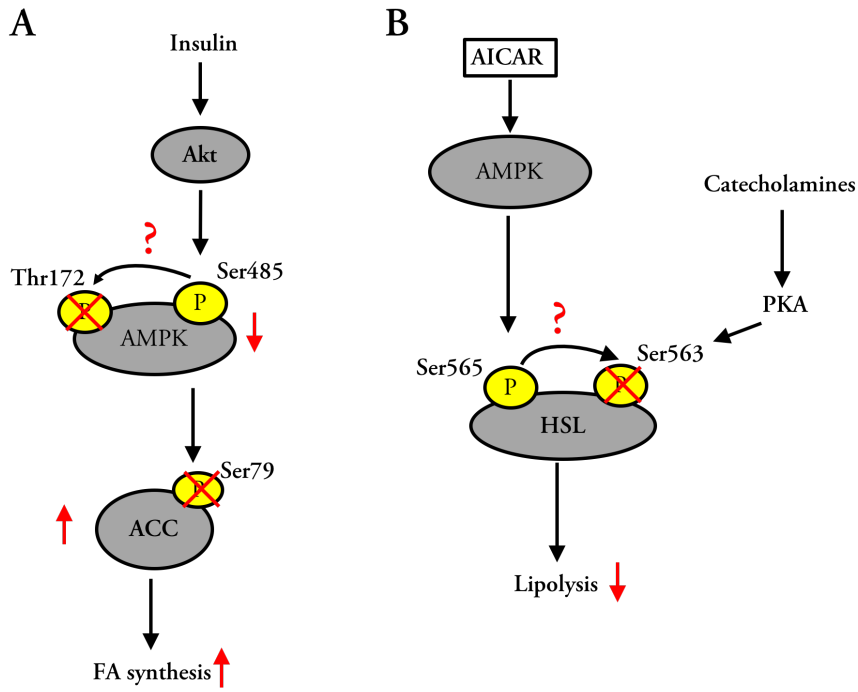

Figure 5. Possible involvement of AMPK in the regulation of FA synthesis (A) and lipolysis (B).
(A) Stimulation of adipocytes with insulin leads to the inhibition of AMPK activity and is associated with increased phosphorylation of AMPK at Ser485. This phsophorylation at Ser485 is proposed to be involved in insulin-induced inhibition of AMPK activity, leading to reduced phosphorylation of the AMPK downstream target ACC at the inhibitor site Ser79, thereby inducing FA synthesis. However, if Ser485 is a requirement for the insulin induced inhibition of AMPK is not know and has been studied in our **Paper IV**. (B) Studies with AICAR propse that AMPK activation leads to inhibition of HSL via Ser565 phosphorylation. Phosphorylation at Ser565 has been suggested to counteract the catecholamine-induced, PKA-dependent HSL Ser563 phosphorylation thereby leading to inhibition of HSL and reduced lipolysis. Effets of the more specific ADaM site activators on lipolysis were investigated in **Paper I**.

A more recent *in vivo* study with inducible adipocyte specific AMPKα1/α2 deficient mice supported an inhibitory effect of AMPK on stimulated lipolysis, via phosphorylation of HSL Ser565 and prevention of Ser563 and Ser660 phosphorylation [65]. On the other hand, this study revealed a stimulatory effect of AMPK activation on basal lipolysis via an increased, activating phosphorylation of ATGL Ser406, which is also in line with the lean phenotype of these mice compared to controls and the importance of ATGL in basal lipolysis [65, 166, 167]. In addition, the data obtained

from AMPKα1/α2 mice suggests suppression of adipocyte FA oxidation and energy usage [65]. This study was also the first to show the effect of an AMPK activator of the newer generation, A-769662, on adipocyte lipolysis, which confirmed a stimulatory and inhibitory effect on basal and stimulated lipolysis, respectively, in mice. However, in the same year, Mottillo et al. published a study employing inducible adipocyte specific β1 and β2 k/o mice claiming that AMPK does not affect adipocyte lipolysis or HSL phosphorylation and that the inhibitory effect of AICAR is AMPK-independent [116].

AIMS

The **overall aim** of this work was to investigate the role of AMPK in the regulation of adipocyte metabolism as well as to assess the regulation, expression and activity of AMPK itself in adipocytes with implications for insulin resistance/T2D.

Specifically, this involved the investigation of:

- the effect of AMPK activation by the ADaM site activators A-769662 and 991 on lipolysis and glucose uptake in different adipocyte models (**Paper Ⅰ and II**)
- the expression and activity of AMPK in different adipocytes models and in relation to BMI in humans (**Paper III)**
- the mechanism(s) underlying the insulin-induced inhibition of AMPK (**Paper IV**)

METHODOLOGY

Adipocytes

For the work presented in this thesis, adipocytes from different origin were employed, including mature primary human and rodent adipocytes as well as *in vitro* differentiated, adipocytes in culture. Whenever possible, human adipocytes were included in the studies but due to limited access this was not always possible. Furthermore, to be able to compare our results to previously published data, which was mainly obtained from primary rodent or cultured adipocytes, we relied on these models to validate our methods and results before moving on to using primary human adipocytes.

Mature primary adipocytes

Primary adipocytes, were isolated from adipose tissue by collagenase digestion and subsequent washing to separate the adipocytes from components of the SVF. The separation of the adipocytes from the SVF is greatly facilitated by the unique property of containing a unilocular lipid droplet making up most of the volume of the adipocytes. While the SVF sinks to the bottom, the adipocytes float to the top.

For stimulation, the cell amount was determined via lipocrit measurement and the cells were diluted in incubation buffer to a concentration suitable for the respective assay. The cell suspension was then distributed equally to the incubation vials. In order to achieve an even distribution of the cells among the vials, it is important to ensure an even cell suspension. This even distribution of cells is of special importance in assays in which no protein amount is determined and no other normalization is performed. Additionally, when determining the cell amount via lipocrit, the actual cell numbers are not determined, which means that due to the heterogeneity of cell size, the number of cells can vary in between experiments. To circumvent this issue, it would be possible to determine the protein amount and normalize accordingly or count the cells and prepare cell suspensions with an equal number of cells for each experiment. However, by determining the cell number, cell size is not taken into consideration, which could also affect cell function and/or metabolism and thereby the results.

Rodent adipocytes

Adipocytes were isolated from epididymal adipose tissue of male Sprague Dawley rats (6 weeks old) and C57BL/6J mice (8-12 weeks old). The epididymal adipose tissue is commonly used when working with white adipocytes from rodents mainly due its abundance and accessibility. However, a drawback with the use of this fat depot is that there is no corresponding depot in humans. Therefore, one needs to be careful when wanting to translate the results obtained from rodent models to human subjects, which is also the reason for including human adipocytes in our studies whenever possible. Furthermore, the studies were limited to the use of male rodents which leads to biased results especially as it is known that distinct gender differences exist. An advantage with using animal models is that are bread and housed under controlled conditions which leads to much lower interindividual variation as in comparison to cells from human subjects (see below).

Human adipocytes

Human adipocytes were isolated from abdominal subcutaneous adipose tissue originating from subjects who underwent either reconstructive surgery or laparoscopic cholecystectomy, and is available to us through collaborations with the Skåne University Hospital in Malmö. The vast majority of this material is obtained from women. Cells from human subjects exhibit a much larger interindividual variation and therefore, a larger number of experiments, compared to rodent cells, is required to be able to draw a conclusion. Furthermore, the subjects included were not selected based on any specific criteria, which means that they are not matched according to factors like age or any kind of lifestyle factors. However, this great variation between the individuals can also be seen as an advantage as the effects observed in such heterogeneous groups are likely to be very robust. A drawback with the material we obtain is that we are not in control of any steps regarding the handling of the tissue until it arrives at the laboratory, which could cause additional variation between the samples. Furthermore, our supply of human tissue is limited and quite irregular, meaning that it is not possible to rely on the availability of human material. However, the access to human adipose tissue constitutes a valuable resource as it allows us to perform *in vitro* investigations on human material resembling the *in vivo* situation as best as possible.

In vitro differentiated 3T3-L1 adipocytes

The 3T3-L1 cell line is a well characterized and most commonly used cell line for the analysis of adipocyte physiology in culture. The cells are differentiated from fibroblast-like preadipocytes of murine origin with the help of an adipogenesis-inducing cocktail inducing the expression of lipogenic genes and the accumulation of lipids. In

comparison to mature, primary adipocytes, 3T3-L1 adipocytes contain many smaller lipids droplet rather than one big droplet. An advantage of the use of cultured adipocytes is that the variation is in general low due to the standardized conditions and that the availability of cell lines also constitutes a possibility to reduce the number of experimental animals used. However, a drawback with cultured cells is that the environment they are grown in does not resemble physiological conditions. Moreover, when using *in vitro* differentiated cells one never achieves a full differentiation of cells in cultures, meaning that in the case of the adipocytes there are always also some preadipocytes present which differ in their biological properties and could influence the obtained result.

Pharmacological activators/inhibitors

AMPK activators

The aim of **Paper I and II** was to evaluate the effects of AMPK activation on adipocyte metabolism, which so far mainly had been investigated with the rather unspecific AMPK activator AICAR. To re-evaluate the effects of AMPK activation on adipocytes, we employed two more specific AMPK activators of a newer generation, A-769662 and 991, which both bind the AMPK ADaM site. Despite the fact that these activators are more specific towards AMPK than AICAR, both of them also affect other kinases as shown in *in vitro* kinase screens [168, 169]. Therefore, one still has to be careful when interpreting the results as observed effects could still AMPK-independent and the results should be verified e.g. by the use of genetic models. In order to minimize the likelihood of off-target effects, it is important to carefully determine a suitable concentration and incubation time, which is sufficient to achieve a robust activation.

Inhibitors

Additionally, in **Paper IV,** different enzyme inhibitors were employed to investigate mechanisms involved in the regulation of AMPK as well potential downstream signaling events. The employment of pharmacological inhibitors constitutes a great possibility to investigate the importance of specific proteins without having to use knockout models with potential compensatory mechanisms and severe cellular alterations.

Akt inhibitor MK-2206

MK-2206 is an Akt inhibitor of the newer generation, which displays improved potency and reduced off-target effects compared to the previously used inhibitor

Akti1/2 [170]. Similar to what was observed with Akti1/2, MK-2206 still exhibits Akt-independent inhibitory effects on adipocyte glucose uptake [170]. However, this Akt-independent effect was only observed at high MK-2206 concentrations (10 μM) and was very modest compared to the off-target effect observed with Akt1/2. Taken together, this means that MK-2206 constitutes a useful tool for the investigation of the role of Akt in adipocytes when used at low concentration.

PDE inhibitors

For the investigation of the involvement of PDEs, specifically PDE3 and PDE4 which are the major PDE families expressed in adipocytes, in the inhibition of AMPK the PDE inhibitors Rolipram (PDE4 inhibitor) and OPC3911 (PDE3 inhibitor) were used.

Western blotting

Western blotting is a commonly used method for the analysis of protein expression and modifications. Throughout the studies presented here, this method was mainly used to analyze protein phosphorylation but also for protein expression analysis (subunit expression) and validation of immunoprecipitation efficiency. The method itself and the quality of the result is greatly dependent upon the availability of antibodies specifically recognizing the protein or phosphorylation site of interest. For the western blots presented in this thesis, commercially available as well as custom-made antibodies were employed. A drawback with western blotting is that the obtained signals are dependent upon many factors like antibody dilution, incubation and washing time, which makes it difficult to compare the signals of different runs. In order to correct for this, we normalized the signals to an internal control, which was set to 100 %, on each membrane. However, performing this kind of normalization entails issues with statistical analysis, which relies on variation. A possibility to circumvent this problem would be to include the same standard sample for each run, to normalize for differences in the signal strength between the different runs. This approach was used for the comparison of protein expression and phosphorylation between human subject presented in **Paper III**. In this study, it was important to normalize the signals of different runs as the main aim was to compare expression between different subjects which could not be run on the same gel. In **Paper I, II + IV** however, we were interested in the fold change induced by treatment which makes the expression as % of a control useful. To still analyze this kind of normalized data a one-sample t-test can be employed. Furthermore, it also important to keep in mind that western blotting constitutes a semi-quantitative method and that no conclusion regarding absolute

protein amounts or phosphorylation can be made but rather relative changes in relation to a control. Therefore, it is also important to include appropriate loading controls.

Adenoviral transduction

To investigate the role of AMPK Ser485 phosphorylation in the regulation of AMPK activity, we overexpressed the phosphorylation mutant Ser485Ala (not phosphorylatable) and Ser485Asp (mimicking permanent phosphorylation) with the help of adenoviral vectors. The basis for adenoviral transduction is the expression of the coxsackie and adenoviral receptor on the cell surface. Important to consider is that the endogenous protein is still expressed in the cell, which means that in order to mask the endogenous protein, sufficient overexpression needs to be ensured. Additionally, to rule out potential alterations caused by the virus transduction and not the expression of the specific protein, an appropriate control needs to be used, like cells transduced with virus expressing an empty vector. Furthermore, it is also important to keep in mind that a strong overexpression does not resemble the physiological situation.

Immunoprecipitation

In **Paper III**, immunoprecipitation (IP) of specific AMPK subunit isoforms was used to investigate the subunit composition of the AMPK complex as well as their respective contribution to total AMPK kinase activity in adipocytes. The antibodies used in this study were custom-made (α1 and β2) or purchased commercially (β1). In order to be able to draw the correct conclusions from the obtained results, it is important to have an antibody with high specificity towards the wanted isoform of the protein of interest available. A possibility to evaluate this antibody specificity in the case of kinases is to perform a comparative analysis of kinase activity (see below) with material deficient in the respective protein isoform which was performed for verification of our in-house AMPKα1 antibody. Specificity of the respective β-isoform antibodies was assessed via IP and subsequent western blotting with a β1/β2-pan-antibody on the remaining lysate and the immune-supernatants. Furthermore, it is important to ensure efficient IP, which can be verified with the help of e.g. western blotting with the immuno-supernatant remaining after IP.

In vitro kinase activity

In vitro kinase activity measurement constitutes a quantitative method for the assessment of kinase activity. The method is based on the incorporation of radioactively labeled ATP into a substrate peptide. However, to able to measure the specific activity of the protein of interest, prior IP with a specific antibody is required, as well as suitable substrate peptide. Antibody specificity can be verified via comparative analysis with respective k/o material, as described previously. Furthermore, to ensure linearity, it is also important to carefully evaluate the amount of protein lysate. As the suitable amount can vary depending on the origin of the protein lysate (rat, mouse, human) this was assessed prior to experiments with the lysates of different origin. A drawback with the use of *in vitro* kinase assays is that changes in activity which are mainly based on allosteric effects are difficult to detect as the bound compound might be released from the protein during sample preparation. Furthermore, as this assay involves work with ^{32}P, special caution and protective equipment is required.

Glucose Uptake

The uptake of glucose into adipocytes in **Paper II** was investigated with the two glucose isotopes ^{14}C-D-glucose (primary cells) or 2-[^{3}H]-deoxy-D-glucose (3T3-L1 adipocytes), with the latter not being able to be metabolized within the cells. In the case of primary adipocytes, the cells were separated from the labeled glucose-containing incubation medium by centrifugation through oil to stop the reaction. In contrast, as 3T3-L1 adipocytes are attached to the culture dish, the reaction could be stopped by removing the buffer and washing the cells. To be able to determine the amount of labeled glucose associated with the cells, but which has not been taken up, cells treated with cytochalasin B, a potent inhibitor of glucose transporters, were included as a negative control. The glucose taken up by the cells was determined via detection of the present radioactivity by liquid scintillation counting.

Lipolysis

During lipolysis, TAGs stored in the adipocyte lipid droplet are broken down to glycerol and FAs. To measure lipolysis in our experiments, we took advantage of the glycerol released to the medium, which through an enzymatic reaction can be converted to a dye [171]. This allows the measurement of lipolysis via fluorescence measurement. The reason for measuring glycerol in contrast to FAs is that the FAs might be re-esterified meaning that the released FAs do not necessarily reflect the degree of lipolysis.

Over the course of time in which the lipolysis experiments in **Paper I + IV** were performed, two different assays were used. The reason for this is that the assay we started off with contained hydrazine. Due to the health risks associated with hydrazine, this assay was replaced with a commercially available hydrazine-free kit. However, the method was not changed within a set of experiments as the distinct read-outs would make the comparison of the obtained results impossible.

De novo FA synthesis

FA synthesis was measured using ^{3}H-acetate [172]. Upon entry into the cell, acetate is converted into acetyl-CoA, the main building block of FAs. This allows the assessment of FAs via the measurement of radioactivity incorporated into the lipids. The cells are incubated in the presence of ^{3}H-acetate and the reaction is stopped by the addition of a water-insoluble scintillation fluid allowing the lipid phase, containing the ^{3}H-labeled newly synthesized lipid, to separate from the aqueous phase. Thereby, radioactivity specifically in the lipid phase can be determined [173].

Nucleotide measurement

For the analysis of potential changes in cellular energy levels in response to insulin in **Paper IV,** the nucleotide content (AMP, ADP and ATP) in our samples was measured by HPLC in collaboration with Mark Rider (UCL, Brussels; Belgium). However, as at that time no protocol for the measurement of nucleotides specifically from adipocytes was available, the first step was to establish a suitable sample preparation protocol. The main challenge here was to produce samples containing as little remaining free fat as possible as this could first of all interfere with the measurements, but also harm the equipment. Furthermore, as changes in nucleotide levels are quickly reversed, it is also crucial to ensure a quick lysis of the cells. However, once able to produce sample of good quality, the big advantage of the HPLC measurement is the great sensitivity, allowing an accurate measurement of also very low nucleotide levels, especially AMP, which constitutes an advantage in comparison to commercially available kits.

RESULTS AND DISCUSSION

Effects of AMPK activation on adipocyte metabolism (Paper I and II)

Key findings

Lipolysis (Paper I)

- AMPK in primary adipocytes is significantly activated by the ADaM site activators A-769662 and 991 (first study showing this)
- AMPK activation has no anti-lipolytic effect in human adipocytes.

Glucose Uptake (Paper II)

- Adipocyte glucose uptake is differentially affected by A-769662 and 991: while A-769662 induces a decrease in insulin-stimulated glucose uptake, 991 shows no effect.
- The inhibitory effect of A-769662 on adipocyte glucose uptake is likely to be AMPK-independent and possibly involves direct inhibition of the glucose transporters GLUT4 or their trafficking.

Although dysregulation of adipose metabolism has also been shown to play an important role in obesity and metabolic disorders [21, 26, 174, 175], effects of AMPK activation have only been studied to a minor degree in adipocytes and mainly with the rather unspecific activator AICAR [52, 64, 111, 155, 157, 163, 176, 177]. Therefore, we investigated the effect of AMPK activation on adipocyte metabolism i.e., lipolysis (**Paper I**) and glucose uptake (**Paper II**) with more specific activators of a new generation.

The effect of AMPK activation on lipolysis differs among species (Paper I)

To re-examine the previously proposed anti-lipolytic effect of AMPK activation in adipocytes [64, 65, 111, 163, 176], we employed the two ADaM site activators A-769662 and 991 [87, 118].

Isolated primary human, rat or mouse adipocytes were pre-treated with increasing concentration of either A-769662 or 991. Additionally, to be able to compare to previously published results, cells pre-treated with AICAR were also included. Subsequently, lipolysis was induced via stimulation with isoproterenol (iso). Post-stimulation, the incubation medium was collected, and cells were lysed for later western blot analysis. For the measurement of lipolysis, the amount of glycerol released into the medium was determined. The workflow is depicted in Figure 6.

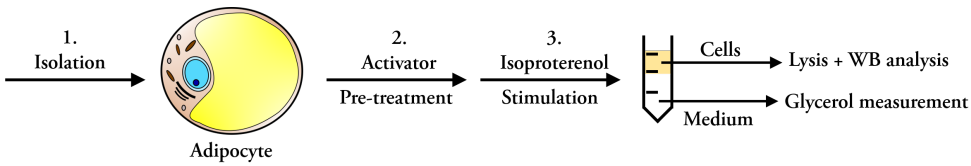

Figure 6. Workflow for the anlysis of lipolysis and HSL phosphorylation in Paper I.
Isolated adipocytes were treated with with either of the activators AICAR, A-769662 or 991 prior to stimulation with isoproternol. Subsequently, the incubation medium was collected for the assessment of lipolysis via meaurement of glycerol levels and cells were lysed for phosphorylation analysis by western blotting.

In line with previous studies, we observed a consistent inhibition of iso-stimulated lipolysis in human as well as rodent adipocytes with AICAR. Interestingly, with A-769662 or 991, no or only a minor anti-lipolytic effect was detected in human or rat adipocytes. Considering, that both ADaM site activators were largely without effect on stimulated lipolysis in human and rat adipocytes it was of great importance to ensure the functionality of our assay. Therefore, we also included mouse adipocytes in this study, as A-769662 had already previously been used in these cells to study the effect on lipolysis [65]. In line with this previous study, we found iso-stimulated lipolysis to be significantly reduced by A-769662 in mouse adipocytes. However, our data shows that AMPK is efficiently activated by A-769662 as well 991, shown by increased AMPK phosphorylation at the activity regulating site T172 as well as the downstream targets Raptor at S792 and ACC at S79 and increased *in vitro* kinase activity. Moreover, we were able to consistently reproduce the reported anti-lipolytic effect of AICAR in all adipocyte models. Taken together, this demonstrates that the lack of effect we observed in human and rat adipocytes was not caused by inefficient activation or the inability of our assay to detect changes. Therefore, we conclude that AMPK activation by ADaM site activators indeed does not inhibit lipolysis in human and rat adipocytes.

Altogether, the obtained results indicate that the three AMPK activators used in this study have differential, species-specific effects on adipocyte lipolysis. Knowing that each of these activators possesses a unique AMPKβ isoform preference, it might be possible that the differential effects we observed were caused by these specific preferences [87, 178, 179]. However, AICAR, activating all AMPK complexes regardless of the identity of the β-subunit and A-769662, showing a preference for β1-containing complexes, have shown similar effects in mouse adipocytes although these display the highest β2 expression in relation to β1. Furthermore, AICAR and A-769662 have shown distinctly different effects in rat adipocytes, which almost only express β1. Additionally, despite the comparable AMPKβ isoform pattern in human and mouse adipocytes, the human lipolysis data was more reminiscent of that in rat. Therefore, we believe that the differences between the activators and species are not likely caused by the β-subunit preferences and propose that the effects observed with AICAR are AMPK-independent. However, the data on β-isoform expression presented in **Paper I** is limited to the analysis with a AMPKβ-pan-antibody, which does not allow for a quantitative comparison. Therefore, we have performed a more detailed and rigorous analysis of the β-isoform expression in adipocytes from different species, including human, in **Paper III**.

The regulation of HSL and lipolysis is species dependent (Paper I)

To further assess the mechanism behind the species-specific differences observed with the different activators, we investigated the effect on the phosphorylation of HSL.

Cell lysates, prepared after collection of the medium for lipolysis analysis (Figure 6), were subjected to western blotting and phosphorylation of HSL at the sites Ser563, Ser565 and Ser660 was monitored.

For mouse adipocytes, our results showed a significant reduction of iso-stimulated Ser563 phosphorylation and induction of Ser565 phosphorylation with AICAR and A-769662. This observation, together with the reduced iso-stimulated lipolysis we have seen in these cells, is in line with the suggested inhibitory effect of AMPK-dependent HSL Ser565 phosphorylation [63-65]. Furthermore, similar analysis of HSL phosphorylation in rat adipocytes showed that in accordance with the lack of effect on lipolysis, neither A-769662 nor 991 induced significant changes in HSL Ser563 or Ser565 phosphorylation. However, in primary human adipocytes, pre-treatment with 991 induced significant changes in HSL phosphorylation similar to the ones observed with A-769662 in mouse adipocytes, although lipolysis was only altered to a minor degree and only with a rather high 991 concentration. Based on previous reports, suggesting HSL Ser660 as the main site involved in the regulation of HSL kinase activity [180], we also investigated possible changes at this site in response to AMPK activation in our adipocyte models. Interestingly, we observed an increase in iso-stimulated HSL Ser660 phosphorylation in human adipocytes, while no significant

changes at this site were observed in mouse or rat adipocytes in response to A-769662 or 991. Our interpretation of these results is that HSL Ser660 phosphorylation plays a key role in the regulation of HSL activity in human adipocytes by "dominating" over the reduced Ser563 phosphorylation, resulting in unaltered HSL activity. Taken together, the provided HSL data suggests several interspecies differences also in the phosphorylation/regulation of HSL.

AMPK activators A-769662 and 991 exert differential effects on glucose uptake in adipocytes (Paper II)

After having assessed the effect of AMPK activation on lipolysis, we investigated the effects on adipocyte glucose uptake in **Paper II**. The data available so far suggests a stimulation of basal glucose uptake and inhibition of insulin-stimulated glucose uptake in 3T3-L1 adipocytes while in primary adipocytes both, basal and stimulated glucose uptake, were shown to be suppressed [52, 155, 157, 177]. However, these results were all obtained with AICAR. An inhibitory effect of AMPK activation in adipocytes would be in contrast with the stimulatory effect observed in muscle. Therefore, it is of great interest to assess whether this discrepancy mirrors a real difference or whether it caused by some AMPK-independent effect of AICAR in adipocytes.

To investigate the effect of the more specific AMPK activators A-769662 and 991 on adipocyte glucose uptake, isolated primary human and rodent adipocytes or cultured 3T3-L1 adipocytes were treated with either of the two ADaM site activators, or AICAR, prior to further stimulation with insulin. Subsequently, glucose uptake was measured via the uptake of ^{14}C-glucose (primary cells) or [^{3}H]-2deoxy glucose (3T3-L1). See Figure 7 for workflow. Furthermore, phosphorylation of Akt and Akt downstream targets, focused on AS160, was assessed. In order to investigate AMPK specificity of potential effects observed with ADaM site activators, we employed adipocytes from a AMPKβ1 Ser108Ala mutant mouse model, in which AMPK cannot be activated by ADaM site activators.

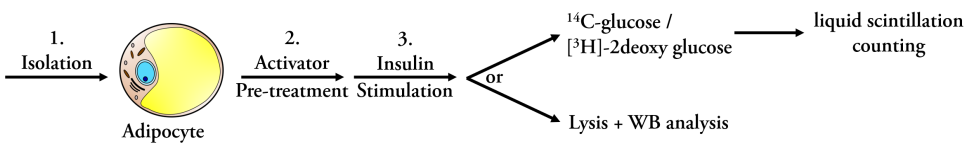

Figure 7. Workflow for the analysis of AMPK activity and insulin signaling by western blotting as well as glucose uptake in Paper II. Isolated adipocytes were treated with with either of the activators AICAR, A-769662 or 991 prior to stimulation with insulin. Subsequently, cells were lysed for phosphorylation analysis by western blotting or labled glucose was added to measure glucose uptake.

In line with previous publications, our results showed that AICAR induced a consistent decrease in basal as well as insulin-stimulated glucose uptake in primary rat adipocytes while in 3T3-L1 adipocytes only stimulated glucose uptake was reduced [52, 155, 157, 177]. Furthermore, we observed that AICAR also reduced glucose uptake in primary human adipocytes. When treating the cells with the ADaM site activators, A-769662 caused a significant, dose-dependent reduction of glucose uptake in all adipocyte models. In contrast, 991 did not affect glucose uptake in any of these adipocyte models. The fact that the activators show comparable effects in rat and human adipocytes even though the human cells appear to express more equal amounts of β1 and β2 compared to primary rat adipocytes ([181] + unpublished data in **Paper III**), suggests that the differential effects obtained are not due to specific AMPKβ isoform preference of the activators. Therefore, it does not seem likely that the isoform preferences play a role in for effect of the activators.

The inhibitory effect of A-769662 is not explained by altered insulin signaling.

To further investigate possible mechanisms underlying the inhibitory effect of A-769662, we examined potential effects on Akt phosphorylation/activity, which is required in the regulation of insulin-stimulated glucose uptake [39]. In rat adipocytes, neither A-769662 nor 991 induced any changes in the phosphorylation of Akt at the sites Ser473 or Thr308. Insulin-stimulated phosphorylation of the downstream targets AS160 at Thr642 or GSK3α/β at Ser21/Ser9 was, if anything, even increased in response to both activators. Based on this, we conclude that changes in Akt signaling are not likely to explain the differential effects of A-769662 and 991 on glucose uptake. However, AS160 is phosphorylated at several other sites in addition to Thr642, that were proposed to be involved in the regulation of AS160 activity and trafficking of the glucose transporter GLUT4 [42, 43, 182]. Therefore, a more detailed analysis of AS160 phosphorylation at the sites Thr642, Ser341, Ser588, and Ser751, which are all phosphorylated in response to insulin, was performed in 3T3-L1 adipocytes. Treatment of the cells with A-769662 tended to increase Ser588 and significantly decreased Ser751 phosphorylation, while no changes at the other sites were detected. However, changes at these sites did not affect the binding of AS160 to 14-3-3, which is thought to be mainly regulated by Thr642 and Ser341 phosphorylation. These results indicate that the decreased glucose uptake by A-769662 is not mediated by changes in AS160 phosphorylation.

Effect of A-769662 on glucose uptake is likely to be AMPK-independent

The lack of effect with 991 on glucose uptake suggested that the observed inhibition with A-769662 might be AMPK-independent. To examine this assumption, we employed primary adipocytes isolated from AMPKβ1 S108A knock-in mice. Western blot analysis of pAMPK T172 as well as pRaptor S792 and pACC S79 in cell lysates from WT and S108A mice confirmed largely abolished AMPK activation in response

to A-769662 in S108A cells. Interestingly, measurement of insulin-stimulated glucose uptake revealed an equal, dose-dependent inhibition by A-769662 in WT as well as S108A mice. This result clearly demonstrates that AMPK activation is not required for the inhibition of glucose uptake by A-769662.

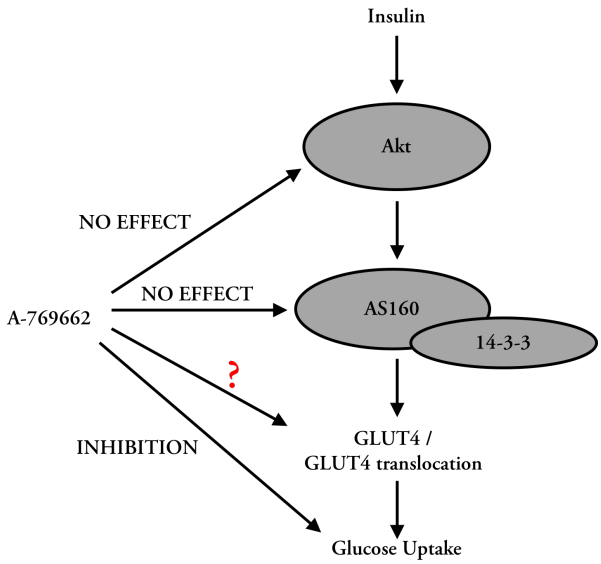

Figure 8. AMPK induces an AMPK-independent inhibition of adipocyte glucose uptake – possible underlying mechanisms.
In our study presented in **Paper II** we found an AMPK independent inhibition of adipocyte insulin-stimulated glucose uptake. Further investigations on the underying mechanism revealed no alterations in insulin signaling on the level of PKB activity or in the association of AS160 with 14-3-3 that could explain the inhibition of glucose uptake. However, our results suggest that A-769662 might affect glucose uptake through direct effects on GLUT4 itself or its trafficking.

Possible mechanisms by which A-769662 might inhibit glucose uptake include direct effects on trafficking of GLUT4, meaning the fusion and/or formation of GLUT4 vesicles or inhibition of the actual transporter. Indeed, by taking advantage of the short incubation time with ^{3}H-labled glucose in 3T3-L1 adipocytes, we could show that the addition of A-769662 together with the labelled glucose for 3 min was sufficient to decrease insulin-stimulated glucose uptake to a level corresponding to basal, non-stimulated glucose uptake. Considering this rapid response, it is quite possible that A-769662 directly affects GLUT4 and/or its trafficking. Additionally, the observation that the removal of A-769662 before measurement of glucose uptake could not reverse the inhibition points towards a strong interaction A-769662 and its target. Investigated in suggested pathways involved in the inhibition of glucose uptake by A-769662 are presented in Figure 8.

As an additional aspect, our study also highlights the necessity of a careful evaluation of effects observed in response to activators. Although A-769662 and 991 activation AMPK through the same mode of action they showed distinct effects on glucose uptake. As the inhibitory effect of A-769662 turned out to be an AMPK-independent effect this might suggest that 991 is more specific AMPK activator than A-769662 which is also in line with previously performed *in vitro* kinase screens in which 991 showed less effects on other kinases than A-769662 [168, 169]. However, our study only provides a snapshot and effects observed with either activator will have to be validated with suitable controls

AMPK activation in adipocytes — possible consequences

In **Paper I + II** of this thesis we demonstrate that the earlier reported inhibitory effects of AMPK activation on adipocyte metabolism i.e. lipolysis and glucose uptake are likely to be AMPK-independent (Figure 9). However, as cellular energy homeostasis is largely dependent upon AMPK it is of great or even greater importance to also report these kinds of "negative findings" to be able to exclude any possible, adverse off-target effects induced by a drug. This necessity of a careful evaluation of potential off-target effects is also highlighted by **Paper II**, in which A-769662 was shown to induce a significant reduction of adipocyte glucose uptake in an AMPK-independent manner.

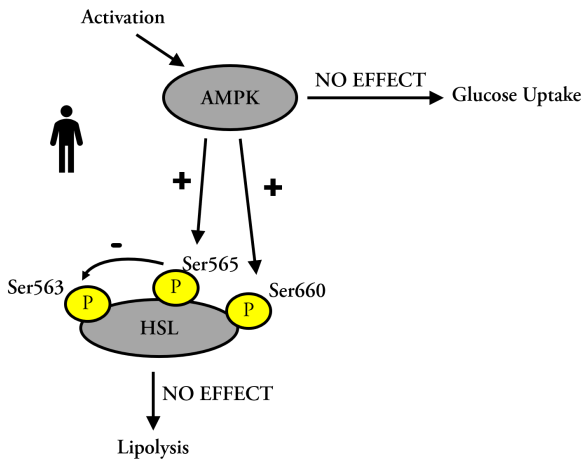

Figure 9. Effect of AMPK activation on lipolysis and glucose uptake in human adipocytes – Conclusions from Paper I + II.
Our finding from **Paper I** showed that AMPK activation induced the phosphorylation of HSL at Ser565 as well as Ser660 which was accompanied by a reduction in HSL Ser563 phosphorylation. In summary, this did not lead to changes in stimulated lipolysis. In **Paper II** we found that AMPK activation does not affect adipocyte glucose uptake

Based on our studies in adipocytes, no findings were made that would compromise the suitability of specific AMPK activators as potential drugs for the treatment of metabolic disorders. However, this does not imply a recommendation, which could only be made on condition that the effects in further tissues have been carefully evaluated to exclude not only undesirable but also unnecessary side effects. Moreover, with our studies we have only assessed the effects of AMPK activation on adipocyte glucose uptake and lipolysis. As mentioned in the introduction, adipocyte FA synthesis also play an important role in maintaining metabolic health and insulin sensitivity. The role of AMPK in the regulation of adipocyte FA synthesis is only poorly studied so far. However, our study presented in **Paper IV** provides evidence for a regulatory role of AMPK on insulin-stimulated FA synthesis. Importantly, to be able to make final conclusion on the suitability of an AMPK activator as a drug it is important to assess its effects in the context of the whole organism i.e. taking the interplay between cells and tissues in consideration for which *in vivo* studies are necessary. Taken together, with our current knowledge, it is not possible to make a definite statement on the consequences of pharmacological AMPK activation in relation to the treatment of insulin resistance/T2D.

Furthermore, it must be considered that our studies investigated the acute, short-term effects of AMPK activation in adipocytes, meaning that no statement on long-term effects can be made. However, these long-term effects are definitely to be considered if wanting to apply AMPK activators as drugs. An attractive strategy would be to target AMPK specifically in the tissues in which activation has been shown to be beneficial, like muscle and liver. The activation efficiency of the pharmacological AMPK ADaM site-binding activators is highly dependent upon the identity of the AMPKβ isoform[87, 118, 169]. This, together with the fact that human muscle and liver tissue express high levels of the β2 isoform compared to for example adipocytes ([183] + unpublished data from **Paper III**), suggests that the activation pattern of ADaM site activators could be narrowed down by the usage of β2 specific activators. However, the development of these β2 activating compounds has proven to be challenging and more recently developed activators like PF-06409577 or MK-8722 also show a preference towards β1-containing complexes [184, 185]. The most promising activator with possible specificity for skeletal muscle is SC4 [120].

In addition to aiding the prediction of how useful AMPK activators are with regards to the treatment of insulin resistance/T2D, our studies contribute to increased understanding about the actual physiological role of AMPK in adipocytes. What are the effects of AMPK activation in response to low energy levels in adipocytes? Our results suggest that alterations in glucose uptake or lipolysis are not involved in restoring cellular energy levels. However, AMPK has been shown to be involved in the regulation of many more metabolic pathways. As already mentioned in the introduction, observed increased β-oxidation is observed in adipocyte in response to long-term AMPK

activation, which could constitute a key mechanism to increase energy production. However, β-oxidation is only poorly studied in adipocytes. Furthermore, to provide energy substrates, e.g. for β-oxidation and also avoid further energy consumption, AMPK-dependent regulation of autophagy and/or pathways involved in cell growth via mTOR, which has been shown previously, could also be important.

AMPKβ-isoform expression patterns vary among adipocyte models (Paper I + III)

Key findings

- Expression and contribution to total cellular AMPK kinase activity of the two AMPKβ-isoforms varies among adipocyte models.
- β1- and β2-containing AMPK complexes make an equal contribution to total cellular kinase activity in primary human adipocytes.

As discussed in **Paper I+II**, the activation efficiency of A-769662 and 991 is highly dependent on the identity of the AMPKβ-isoform. However, this does not only concern the activators we are using in our studies but is rather a general characteristic of ADaM site binding activators, with all of the currently available activators showing a preference for β1-containing complexes [87, 118, 169, 184, 185]. Therefore, to be able to predict the efficiency of these activators in various tissues and to achieve tissue-specific AMPK activation, it is of great importance to assess the relative distribution of β1 and β2 in different tissues, including adipose tissue. In our first study (**Paper I**), we claimed that mouse and human primary adipocytes show a more equal expression of the two isoforms than 3T3-L1 and primary rat adipocytes. These findings were based on western blot analysis with an AMPKβ1/β2 antibody, which might bind the two β-isoform with different efficiencies, preventing us from concluding on the relative β1/β2-expression *within* each cell type. Furthermore, our findings were challenged by a more recent study suggesting that AMPKβ1 is the primary isoform in all of the adipocyte models we analyzed in our study [144]. Based on this, we decided to perform a more extensive and quantitative analysis of the AMPKβ-isoform expression in adipocytes in **Paper III**.

For this purpose, lysates prepared from isolated primary adipocytes or 3T3-L1 cells were subjected to IP with AMPKβ1 and β2-specific antibodies. As a first step, suitability of the used antibodies was thoroughly assessed in order to ensure an isoform-

specific and quantitative analysis. Subsequently, whole lysates, immunoprecipitates, as well as the supernatant collected after IP, were subjected to western blot analysis to determine the AMPKα as well as AMPKβ1 and β2 content. Moreover, we also performed *in vitro* AMPKα-activity measurements after AMPKβ1 and β2-IP. A detailed workflow is presented in Figure 10.

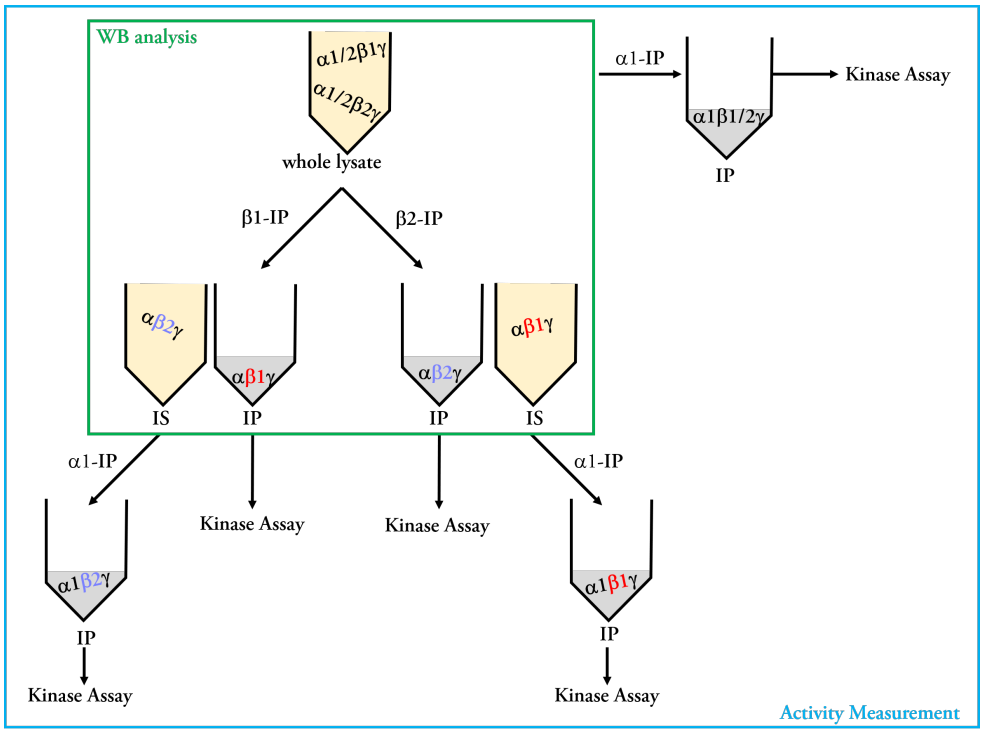

Figure 10. Workflow for the analysis of expression and *in vitro* kinase activity of AMPKβ1/β2-containing complexes via IP.
AMPKβ1- or β2-containing complexes were immunoprecipitated (IPed) with the help of isoform specific antibodies resulting in the IP, containing only the AMPK complexes with the β-isoform against which the antibody was used, and the immuno-supernatant (IS; = lysate after the removal of the respective β-isoform-containing complexes). To assess IP efficiency this immunoprecipitate and supernatant together with the whole lysate were subjected to western blotting. *In vitro* kinase activity was measured from the whole lysate, β1/β2 IPs, as well as α1-IPs of the β-ISs.

Analysis of the expression of the two AMPKβ-isoform in whole lysates with the AMPKβ1/β2 pan antibody in **Paper I** and **Paper III** showed comparable results, i.e. mouse and human adipocytes express more equal amounts of β1 and β2 than 3T3-L1 and rat adipocytes. Furthermore, assessment of the AMPKα levels after IP with a β1 or β2-specific antibody revealed a larger decrease after β1 IP than β2 IP in 3T3-L1 and rat adipocytes, while the decrease of the catalytic α-subunit in mouse and human adipocytes was roughly equal for the β1 and β2 IP. Furthermore, *in vitro* kinase activity measurements showed that in 3T3-L1 and rat adipocytes a larger proportion of total AMPKα activity is associated with the β1- than the β2- isoform. Taken together, these

results imply that 1) AMPKβ-isoform expression levels are in line with the respective contribution to total cellular AMPK kinase activity; 2) AMPKβ1-containing complexes are predominant in 3T3-L1 and primary rat adipocytes, while in human and mouse adipocytes β1- and β2-containg complexes make a roughly equal contribution and 3) that the signal obtained with the used β1/β2-pan-antibody reflects the relation between the two isoforms. This also means that the results we obtain still contradict the earlier published data by Katwan et al. [144]. However, from their published data the IP efficiency of the two β-isoform specific antibodies does not become apparent, meaning that it is not clear whether the total activity IPed with the β antibodies represents total cellular kinase activity. Therefore, one possible explanation for the disparate results could be differences in the achieved IP efficiencies.

Potential dysregulation of AMPK in human obesity (Paper III)

Key findings

- Human obesity is not associated with alterations in AMPK activity or expression in adipocytes from subcutaneous adipose tissue.
- BMI and cellular ACC levels in adipocytes are negatively correlated.

It is well known that obesity and the inability of adipose to store excess energy are associated with an increased risk to develop insulin resistance and eventually T2D [4, 26]. However, whether dysregulation of AMPK, as the master regulator of cellular energy homeostasis, plays a role in this obesity-induced insulin resistance is only studied to a limited extent. Earlier studies conducted with adipose tissue from morbidly obese subjects, however, suggest lower AMPK activity in insulin-resistant, compared to BMI-matched insulin-sensitive subjects as well as increased AMPK activity in subcutaneous adipose tissue post bariatric surgery [146-148, 186]. If these findings are due to changes in the actual adipocytes or are caused by other cell types present in adipose tissue is not known. Therefore, we investigated the association between BMI and AMPK activity in isolated adipocytes from subjects with varying BMI, which is presented in **Paper III**.

Cell lysates, prepared from adipocytes isolated from subcutaneous adipose tissue of 22 individuals (15 female/7male; BMI 38.7 ± 11.4 kg/m^2) were subjected to western blot and phosphorylation levels of AMPKα at Thr172, the AMPK downstream targets ACC Ser79 and Raptor S792 as well as the respective total protein levels were assessed. All

samples were run twice and loaded in a randomized order. Additionally, AMPKα1 *in vitro* kinase activity was measured.

The results of this analysis showed the expected positive correlation between AMPKα Thr172 phosphorylation and *in vitro* kinase activity, as well as AMPKα Thr172 and Raptor Ser792 and ACC Ser79 phosphorylation. In contrast, no association between total AMPKα levels or AMPK activity, measured as Thr172 phosphorylation or *in vitro* kinase activity, and BMI was detected. However, this result does not automatically exclude a potential role of AMPK dysregulation in obesity-induced insulin resistance, as it is possible that the ability of AMPK to respond to signals like insulin (studied in **Paper IV**) is altered, rather than basal AMPK. A possible explanation for the discrepancy between the negative association between AMPK activity and BMI which was proposed earlier and our results might be the fact that our results were obtained from isolated adipocytes while the previous results were obtained from adipose tissue, containing many other cell types. Interestingly, we detected a positive correlation between specific ACC Ser79 phosphorylation (pACC/ACC) and BMI, which was caused by decreasing total ACC levels with increasing BMI. This observation is also in line with the elevated ACC levels associated with weight loss suggested by the data by Albers et al. [147]. As ACC is the rate-limiting enzyme in FA synthesis, this decrease in ACC could constitute an explanation for the impaired FA synthesis in insulin resistance [50, 54]. To investigate this in more detail, it would be interesting to analyze the expression of ACC also in relation to insulin sensitivity. Unfortunately, this information was not available to us for the material analyzed here.

Regulation of AMPK activity by insulin in adipocytes (Paper IV)

Key findings

- AMPK Ser485 phosphorylation is no requirement for insulin-induced inhibition of AMPK kinase activity.
- Insulin stimulation reduces cellular energy levels in adipocytes, constituting an alternative mechanism for the insulin-induced of AMPK kinase activity.
- AMPK Ser485 is highly phosphorylated in response to insulin in primary human adipocytes.

It has been proposed by previous studies that insulin stimulation causes an inhibition of AMPK activity which was also accompanied by increased phosphorylation of AMPK at Ser485. However, it remained unclear if the insulin-induced AMPK Ser485 phosphorylation is required for the inhibition of kinase activity and with that also increased ACC activity and FA synthesis. Therefore, the aim of the study presented in **Paper IV** was to investigate the requirement of AMPK Ser485 phosphorylation for the inhibition of kinase activity, a first step towards the elucidation of how insulin stimulates FA synthesis. Moreover, we assessed whether phosphorylation of AMPK Ser485 also occurs in human adipocytes.

For this purpose, we employed primary rat adipocytes transduced with recombinant adenoviruses encoding AMPK-WT or a non-phosphorylatable Ser485Ala mutant. Transduced cells were stimulated with insulin and AMPK activity was assessed by western blotting as well as by *in vitro* kinase activity measurements. The workflow is depicted in Figure 11.

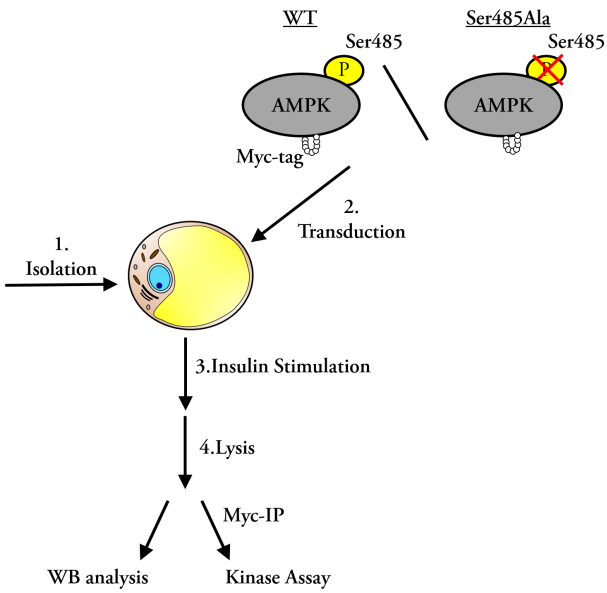

Figure 11. Workflow for the analysis of AMPK activity in AMPK-WT and AMPK Ser485Ala expressing adipocytes in Paper IV. Isolated primary adipocytes were transduced with either AMPK-WT or AMPK Ser485Ala expressing adenoviruses. The transduced cells were subsequently stimulated with insulin and AMPK activity was assessed via western blotting or *in vitro* kinase activity measurement.

Inhibition of AMPK activity by insulin is not dependent on AMPK S485 phosphorylation

To re-evaluate the previously proposed requirement of Akt in the inhibition of AMPK activity by insulin [48], we employed a Akt inhibitor with greater specificity than the earlier used Akti1/2. In line with the earlier results, insulin-stimulation induced a significant inhibition of AMPK activity (measured as decreased phosphorylation of AMPK downstream target as well as AMPK Thr172 phosphorylation and *in vitro* kinase activity) and increased AMPK Ser485 phosphorylation. All of these described effects were prevented in the presence of MK-2206. Furthermore, insulin-induced FA synthesis was also significantly inhibited in the presence of the AMPK activator A-769662, an observation further underlining the involvement of AMPK in the regulation of insulin-induced FA synthesis. Additionally, our data showed a tendency to a negative correlation between AMPK Thr172 and Ser485 phosphorylation. Interestingly, when measuring AMPK activity in AMPK-WT and AMPK-Ser485Ala expressing rat adipocytes, we observed that the mutant was inhibited to a similar degree as the WT by insulin, although phosphorylation at Ser485 was almost completely abolished. This result suggests that, although there is an association between Ser485 and AMPK inhibition, AMPK Ser485 is no requirement for the insulin-induced inhibition of AMPK activity. However, as we observed a tendency towards increased and decreased basal AMPK activity with the Ser485Ala and a Ser485Asp mutant, respectively in comparison to the WT this might imply that Ser485 phosphorylation plays a role for the inhibition of AMPK activity under some conditions. Previous studies employing the AMPK Ser485Ala mutant report a requirement of Ser485 phosphorylation in the inhibition of AMPK in heart and HEK293 cells [82, 102]. An important difference between these studies and ours is that their focus was on the requirement of Ser485 in the prevention of subsequent activation whereas our main focus was on the involvement of Ser485 in inhibiting basal AMPK activity. An additional observation made in our study was that stimulation of adipocytes with insulin prior to the treatment with the AMPK activators reduced the capacity of A-769662 to activate AMPK while AICAR was able to activate AMPK to a similar degree with or without prior insulin stimulation. As the binding site of AICAR at the γ-subunit is in closer proximity to the insulin-induced Ser485 phosphorylation than the ADaM site to which A-769662 binds, this was quite interesting. However, one interpretation could be that it confirms the proposed negligible role of Ser485 phosphorylation in the regulation of AMPK activity by insulin and that Ser485 phosphorylation or the mechanism by which insulin inhibits AMPK activity interferes with the binding of ADaM site binding compounds.

Interestingly, post-publication of **Paper IV,** a study by Pinkosky et al. proposed that long chain fatty acid (LCFA) CoA esters activate AMPK via binding to the ADaM site [105]. Taking this information together with our observation that insulin stimulation

prevents activation by the ADaM site-binding activator A-769662, one could speculate that after a meal, insulin inhibits the release of FAs by inhibiting lipolysis and also induces the synthesis of new FAs potentially via inhibition of AMPK activity and induced ACC activity. As long as insulin levels are high, the newly synthesized FAs are not capable of activating AMPK again to inhibit FA synthesis. Thereby, the storage of lipids is promoted. Conversely, when energy levels are low AMPK becomes activated due to an increase in cellular AMP but also via allosteric activation by the FAs released through induced lipolysis. As a consequence, the synthesis of FAs is inhibited via inhibition of ACC activity by AMPK, and energy production via oxidation is prioritized. Conversely,

Alternative mechanism for the insulin-induced inhibition of AMPK

The next goal was to find alternative mechanisms by which insulin could inhibit AMPK activity. It has been proposed earlier that AMPK is activated by increased lipolysis due to an increase in AMP/ATP caused by energy-demanding re-esterification of FAs. Based on this possible involvement of lipolysis in the regulation of AMPK activity and the known anti-lipolytic effect of insulin we first assessed the role of cAMP signaling/lipolysis in the insulin-induced AMPK inhibition [48, 151-153, 187, 188]. However, neither did basal lipolysis change in response to increasing concentrations of insulin, nor were any changes in AMPK activity detected when inhibiting PDEs and thereby increasing cAMP and lipolysis. Therefore, the idea of an involvement of cAMP signaling/lipolysis in the regulation of AMPK activity by insulin was discarded. As a next step, we then wanted to investigate if insulin stimulation in adipocytes induces a decrease in the cellular AMP/ATP independently of cAMP/lipolysis, which could explain the inhibitory effect of insulin on AMPK activity. Nucleotide measurement from rat adipocytes showed that stimulation with increasing concentrations of insulin indeed induced a dose-dependent decrease in the cellular AMP/ATP ratio, which was shown to be caused by a slight increase in cellular ATP levels and an even stronger decrease in AMP levels. Furthermore, we were able to show that the observed decrease in cellular AMP/ATP ratio is dependent on Akt activity, which is in line with the observed requirement of Akt for the inhibition of AMPK activity and stimulation of FA synthesis. Additionally, we saw that the insulin-induced changes in AMP/ATP ratio occur independently of the increased glucose uptake caused by insulin stimulation. In summary, this indicates that inhibition of AMPK kinase activity in response to insulin is caused by an Akt dependent increase in cellular energy. A summary of the results obtained in **Paper IV** is shown in Figure 12.

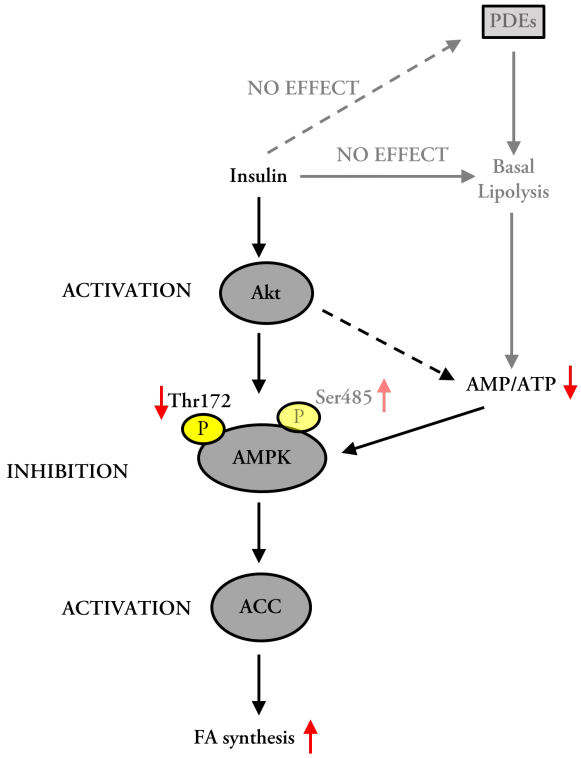

Figure 12. Pathways leading to the induction of adipocyte FA synthesis by insulin - Summary of our results obtained in Paper IV. Our investigations from **Paper IV** showed that although insulin induces a Akt dependent phosphorylation of AMPK Ser485, this phosphorylation is not required for the inhibition of AMPK activity and induction of ACC activity. In the search for alternative pathways, we showed that neither lipolysis nor other PKA-dependent pathways are involved in the insulin-induced inhibition of AMPK activity as insulin stimulation did neither affect basal PDE activity nor lipolysis. Interesting, we found a Akt-dependent decrease in the cellular AMP/ATP ratio in respone to insulin which could possibly explain the inhibition of AMPK activity. Further investigations are required to elucidate the underlying mechanisms.

AMPK Ser485 phosphorylation in primary human adipocytes

In addition to primary rat adipocytes, AMPK Ser485 phosphorylation was also analyzed in primary human adipocytes. Stimulation with increasing insulin concentrations showed a strong induction of Ser485 phosphorylation - a finding which is line with the earlier published data by Albers at al. showing increased Ser485 phosphorylation in adipose tissue during euglycemic clamp [147]. In their study, they also reported decreased AMPK Thr172 phosphorylation along with increased Ser485 phosphorylation. In our study, we observed decreased Thr172 phosphorylation in response to insulin in most subjects analyzed, which resulted in a trend towards a negative correlation between AMPK Ser485 and Thr172 phosphorylation (p=0.0693). However, the effect on Thr172 phosphorylation, and ACC Ser79, varied largely among subjects, with some even showing increased phosphorylation, which resulted in the

changes to be non-significant overall. A possible explanation for the different results obtained might be the fact that again, we analyzed the effect on isolated adipocytes while the study by Albers et al. was focused on adipose tissue composed of many different cell types. Taken together, our results obtained in human adipocytes, are in line with the findings from rat adipocytes, implying that AMPK Ser485 phosphorylation is dispensable for the insulin-induced inhibition of AMPK activity. However, an involvement of Ser485 in the regulation of AMPK activity cannot be entirely excluded. Despite the large variation of the effect of insulin on AMPK Thr172 and ACC Ser79 phosphorylation we observed a decrease at these sites in most of the analyzed subjects, also explaining the tendency towards a negative correlation between Ser485 and Thr172. This observation implicates that insulin-induced AMPK Ser485 phosphorylation is associated with decreased AMPK activity at least in some individuals. Albers et al. also report an improved response in Ser485 phosphorylation post-bariatric surgery indicating a possible association between AMPK Ser485 phosphorylation and obesity/BMI.

SUMMARY AND FUTURE PERSPECTIVES

The studies presented in this thesis have contributed to a better understanding of the role and regulation of AMPK in adipocytes, but also unraveled many unsolved questions that need to be addressed in future studies.

In **Paper I + II**, the focus was on re-evaluating the previously reported effects of AMPK activation by AICAR on adipocyte metabolism, i.e. lipolysis and glucose uptake, for which the ADaM site activators A-769662 and 991 were employed. Based on the obtained results, we propose that neither lipolysis nor insulin-stimulated glucose uptake are affected by AMPK activation and that the inhibitory effects observed with AICAR are likely to be AMPK-independent, at least in humans. However, in **Paper I** we discovered that lipolysis seems to be regulated in a species-specific manner, with HSL Ser660 phosphorylation induced by AMPK activation potentially playing a more important role than Ser563 phosphorylation, at least in primary human adipocytes. So far, HSL Ser660 is only known to be phosphorylated by PKA [60], meaning that the mechanism by which AMPK induces Ser660 phosphorylation remains to be elucidated just like the role of HSL Ser563 phosphorylation. An interesting finding from **Paper II** worth following up was that A-769662 induced an AMPK-independent decrease in the insulin-stimulated glucose uptake, which not only occurred rapidly but also seemed to be caused by a strong interaction. Here it would be interesting to further investigate the exact mechanism by which A-769662 inhibits glucose. One possibility could be that A-769662 affects the translocation of GLUT4 to the plasma membrane, which we are planning to follow up with the help of TIRF microscopy. As a continuation of the studies in **Paper I + II** and to further investigate potential effects of AMPK activation, it would also be interesting to expand our investigations towards further metabolic pathways. This includes the expansion of our study on the role of AMPK in the regulation of FA synthesis presented in **Paper IV,** for which AMPK Ser108Ala mice could be used to assess AMPK-dependence of the effect observed with ADaM site activators. Furthermore, ACC Ser79Ala mice could be employed to investigate the requirement of the AMPK-dependent Ser79 phosphorylation for the regulation of FA synthesis. Additionally, investigations of effects on β-oxidation and autophagy would also be of great interest.

The first part of **Paper III** focused on the expression pattern of the two AMPKβ isoforms in various adipocyte models as well as the contribution of the respective β-isoform containing complexes to total cellular AMPK activity. Our results showed that the β1-isoform is the predominant one in rat and 3T3-L1 adipocytes while in human and mouse adipocytes, β1 and β2 make an equal contribution. These results not only indicate that mouse adipocytes might be the best suited model when wanting to translate the obtained results to human adipocytes but also help to predict the effect of ADaM site activators. To obtain a complete picture, it would be of interest to complement the data with a similar analysis for the three AMPKγ-subunits. Unfortunately, we were only able to analyze the γ1-subunit and that also only by western blotting, due the lack of functional antibodies for IP.

In the second part of **Paper III**, we showed that obesity is not associated with decreased expression or activity of AMPK in adipocytes but revealed a negative correlation between BMI and ACC levels, which might contribute to the reduced FA synthesis observed in insulin resistance and obesity [189, 190]. In this study, samples from 22 human subjects with varying BMI were analyzed. However, most subjects included were obese and it would be interesting to see whether the results we obtained also hold true for lean or overweight subjects with varying BMI. As previous studies showed differences between insulin-sensitive and insulin-resistant BMI matched subjects, it would be interesting to perform a similar analysis of AMPK expression and activity in adipocytes, in relation to insulin sensitivity. As the finding on the relation of the two isoforms in human adipocytes presented in the in the first part of **Paper III**, was based on material of one individual only, it would be interesting to use the material used in the second part here for a more detailed analysis of the expression of the β1- and β2-isoform. Therefore, it would be interesting to see if any inter-individual and/or BMI-related differences regarding the expression of the two β-isoforms exist.

Finally, in **Paper IV**, the regulation of AMPK itself by insulin was in focus. In comparison to what was proposed in previous studies, we showed that AMPK Ser485 phosphorylation is no requirement for the inhibition of kinase activity in response to insulin stimulation in adipocytes. However, we were able to show that insulin induces a Akt-dependent decrease in the cellular AMP/ATP ratio, independent of increased glucose uptake. The mechanism by which this decrease in the AMP/ATP ratio occurs remains unclear. To elucidate the mechanism underlying the insulin-induced decrease in the AMP/ATP ratio it would be interesting to also investigate a potential involvement of insulin-regulated FA synthesis. For this purpose, an inhibitor of FA synthesis like triacsin C could be used. For the investigation of further mechanisms involved in the inhibition of AMPK by insulin, we are planning to assess whether increased glucose uptake is involved. Moreover, as FAs (LCFA-CoA esters) have recently been proposed to be able to induce AMPK activity, it would also be relevant to investigate whether this regulatory mechanism is of importance in adipocytes [105].

If FAs are indeed capable of activating AMPK, one could imagine a negative feedback loop in which insulin induces FAs synthesis which again activates AMPK to inhibit the synthesis of more FAs. As regulation of AMPK by these FAs, and also pharmacological activators, has been shown to be dependent on AMPKβ1 S108 phosphorylation it might also be worth investigating a role of this site in the inhibitory effect of insulin. For this purpose, the already in **Paper III** used AMPK Ser108Ala mice could be employed. Further aspects of this study worth following up are 1) the role of the strongly (by insulin) induced Ser485 phosphorylation, especially as it is also observed in primary human adipocytes. For this purpose, the employment of transgenic AMPK Ser485Ala mice would be useful; 2) the mechanism by which insulin prevents the activation of AMPK by A-769662 (and possibly other ADaM site activators?) and 3) as the ultimate goal of this study is to reveal the mechanism by which insulin induces increased FA synthesis, it would be interesting to employ the ACC Ser79Ala knock-in mouse model to further delineate the mechanism underlying insulin-induced activation of ACC and FA synthesis. As ACC is suggested to be allosterically regulated it would also be interesting to investigate whether insulin stimulation induces changes in citrate levels which could be involved in the induction of ACC activity.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Seit 1975 hat sich die Anzahl der an Fettleibigkeit (BMI≥30kg/m^2) leidenden Menschen mehr als verdreifacht. Die Hauptursache für die Entstehung von Fettleibigkeit liegt in einem Ungleichgewicht von Energiezufuhr und Verbrauch, das heißt es wird mehr Energie zugeführt als verbraucht. Fettleibigkeit stellt einen großen Risikofaktor für die Entstehung von Stoffwechselkrankheiten wie Typ 2 Diabetes (T2D) dar. Bei T2D sind die Zellen des Körpers nicht mehr in der Lage die durch die Nahrung aufgenommene Glukose aus der Blutbahn in die Zellen zu transportieren, was zu einem erhöhten Blutzuckerwert führt. Daher auch die umgangssprachliche Bezeichnung als „Zuckerkrankheit". Doch obwohl Fettleibigkeit ein großes Risiko für die Entstehung von T2D darstellt, erkranken bei weitem nicht alle an Fettleibigkeit leidenden Menschen an T2D. Die genauen Faktoren, die zur Entstehung von T2D beitragen sind komplex und nicht vollständigen verstanden. Sicher ist jedoch, dass eine entstehende Fehlregulation des Fettgewebes eine wichtige Rolle spielt. Fettgewebe besteht zu einem Großteil aus Fettzellen, auch Adipozyten genannt, die Energie in Form von Fett speichern und wenn nötig diese Energie wieder freisetzen können. Kommt es zu einer Fehlregulierung des Fettgewebes ist die Funktion der Fettzellen eingeschränkt und Fett kann nicht mehr eingelagert werden. Stattdessen wird vermehrt Fett freigesetzt und in anderen Organen oder Muskeln abgelagert. Das wiederum kann die Funktion dieser Organe und Muskeln einschränken.

Mit den heute zur Verfügung stehenden Medikament kann T2D gut kontrolliert werden. Die gängigen Therapien haben allerdings häufig Nebenwirkungen, weshalb weiter an neuen Medikamenten geforscht wird. Immer weiter in den Fokus gerückt sind hierbei Aktivatoren der sogenannten AMP-aktivierten Proteinkinase (AMPK). Bei der AMPK handelt es sich um ein Protein, das in jeder Zelle des Körpers verkommt. AMPK ist in der Lage zu erkennen, wenn das Energielevel der Zelle sinkt, sprich der „Treibstoff" fehlt. Ist das Energielevel in der Zelle niedrig, wird die AMPK aktiv und sendet Signale aus die dafür sorgen, dass energieproduzierende Prozesse angeschaltet werden und energiekonsumierende Prozesse gestoppt werden. Die Zelle wechselt vom „Verbraucher/Speicher"-Modus in den „Kraftwerk"-Modus. Steigt der Energielevel wieder wird die AMPK inaktiviert.

In Bezug auf die Behandlung von T2D mit Hilfe von pharmakologischen AMPK Aktivatoren wurde in den letzten Jahren herausgefunden, dass durch die Aktivierung der AMPK eine Senkung der Blutzuckerwerte erreicht werden kann. Der bisherige Forschungsschwerpunkt lagt jedoch auf Muskel- und Lebergewebe. Diese beiden Gewebe tragen einen Großteil zur Regulierung des Blutzuckerlevels bei, da Muskeln den im Blut vorhandenen Zucker verbrauchen, wenn wir uns bewegen, und die Leber Glukose in die Blutbahn abgibt. Obwohl bekannt ist, dass Fettgewebe eine entscheidende Rolle in der Entstehung von T2D darstellt ist nicht viel über den Effekt der AMPK-Aktivierung in Fettzellen bekannt. Aus diesem Grund war das Ziel der hier präsentierten Doktorarbeit die Erforschung der durch AMPK-Aktivierung verursachten Effekte in Fettzellen. Zusätzlich wurde auch die physiologische Regulierung (körpereigene Regulierung, unabhängig von Veränderungen von außen durch z.B. Medikamente) und mögliche Veränderung der AMPK (Aktivität und Vorkommen) durch Fettleibigkeit adressiert.

Die Ergebnisse zeigen, dass die Aktivierung der AMPK weder Auswirkungen auf die Freisetzung von Fett aus Fettzellen hat, noch die Glukoseaufnahme der Zellen einschränkt. Allerdings stellen die Glukoseaufnahme und Fettfreisetzung nur einen Bruchteil der Prozesse dar, die durch die AMPK reguliert und gesteuert werden. Deshalb ist es nötig, in Zukunft noch mehr dieser Prozesse zu erforschen, um die Eignung von AMPK-Aktivatoren für die Behandlung von T2D besser bewerten zu können. Des Weiteren zeigen unsere Ergebnisse, dass es zwar keinen Zusammenhang zwischen AMPK Aktivität und Vorkommen in Fettzellen gibt, aber mit steigendem BMI das Vorkommen eines an der Fettsynthese beteiligten Regulators reduziert ist. Zu guter Letzt, konnten wir in dieser Arbeit zeigen, dass Insulin, welches für die Aufnahme von Glukose aus dem Blut nötig ist, zu einer Erhöhung des zellulären Energielevels, und damit einer Inaktivierung der AMPK führt. Diese Inaktivierung der AMPK könnte im Zusammenhang mit einer erhöhten Fettsäuresynthese zu stehen.

Zusammengefasst, trägt die hier präsentierte Arbeit zu einer verbesserten Kenntnis der AMPK und der durch sie regulierten Prozesse in Fettzellen bei. In Zukunft kann dieses Wissen hoffentlich zu einer besseren Beurteilung von möglichen Nutzen der AMPK in der Behandlung von Krankheiten wie T2D beitragen.

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(Kungsleden; July 2019)

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